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Designing a novel 3-D in-Vitro scaffold to define mechanisms underlying neuronal myelination

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Designing a novel 3-D in-Vitro scaffold to define mechanisms underlying neuronal myelination

by

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Abstract

All nervous system functions in animals require neuronal assembly during development and the ensuing communications between large networks of neurons, which are often difficult to monitor in the intact brain. As such, most labs around the globe have opted to use and explore *in vitro* model systems where neurons are generally grown on two dimensional, plane glass substrates, which limit the ability to decipher fundamental understanding of the mechanisms underlying neuronal growth, polarity and synapse specificity. Most approaches used today employ 2-D models where neurons are cultured on the Poly-D-lysine (PDL) coated substrate which does not mimic the 3-D configuration of the intact mammalian brain – thus limiting a direct comparison between *in vivo* and *in vitro* conditions. Assessing cellular and molecular mechanisms of neuronal myelination are critical to determine how myelination and demyelination processes occur in vertebrate models so as to understand developmental and neurodegenerative diseases such as multiple sclerosis. However, there are no suitable *in vitro* models available to date whereby the process of axon myelination could be studied directly at the level of individual central and peripheral neurons. In contrast to PDL, collagen offers a 3-D structure in which neurons can be suspended in a 3-D configuration, allowing glia to gain access to axonal membrane to exhibit myelination. However, we still lack a reliable 3-D model where mechanisms of neuronal polarity and myelination could be studied at the level of individual peripheral and central neurons. In this study, I designed a 3D substrate comprising of a gelatin base hydrogel with tunable chemical mechanical properties. Using rat Dorsal Root Ganglia Cells (DRG) and their corresponding Schwann cells (SC), I compared and contrasted the effectiveness of GelMA with PDL and Collagen substrates and provide the first direct evidence that the former is more conducive to studying myelination than the later two. Moreover, I also demonstrate that both DRG growth and SC behavior on GelMA resembles to what is seen *in vivo* thus validating further the usefulness of this substrate for future studies.

ACKMOWLEDGMENT

Dedication

To millions of people suffering from MS

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List of Symbols, Abbreviations and Nomenclature

AA	Ascorbic acid
BFGF	basic fibroblast growth factor
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CASPR	Contactin associated protein
CNS	Central nervous system
CSPs	cardiac side population cells
DRG	Dorsal Root Ganglion
DW	Distilled water
E12	Embryonic day 12
ECM	Extra cellular matrix
EDTA	Ethylendiaminaceticacid acid
ErbB1	Epidermal growth factor receptor family (ErbB1, ErbB2)
FBS	Fetal bovine serum
GelMA	Gelatin methacryloyl
GFP	Green fluorescent protein
IGFs	insulin-like growth factors
MAG	Mayelin associated glycoprotein
MBP	Myelin basic protein
MS	Multiple Sclerosis
Na	Sodium
Nav1.6	Voltage gated sodium channel 1.6
NCAM	neuronal cell adhesion molecules
NF	Neurofillament
NGF	Nerve growth factor
NRG1	Neuregulin1
P0-P3	Postnatal 0-3
PCD	programmed death cell

Pen/Strep	Penicillin/Streptomycin
PSC	Precursor scwann cells
RGD	Argenine-Glycin aspartic acid
SAM	substrate adhesion molecules
TGF	Transforming growth factor
TTFG	Fish gelatin
UV	Ultra violet
VGPC'S	Voltage-gated potassium channel

1   Chapter 1: Introduction

1.1   The Nervous System

The nervous system in higher animals is organized into the central nervous system (CNS), which includes the brain, spinal cord, optic, olfactory and auditory systems. The peripheral nervous system (PNS) on the other hand, consists of cranial nerves emerging from the brain, spinal nerves arising from the spinal cord and sensory nerve cell bodies (dorsal root ganglia, DRG) and their process. The CNS and PNS are either connected to or communicate with each other at various levels – either directly through nerves or indirectly via various neuro-hormones and neurotransmitters.

A neuron - which comprises the fundamental unit of both CNS and PNS consist of a cell body and its extensions - including axons and dendrites. Neurons do however, vary in their morphology - from being unipolar to multipolar and exhibit a variety of sizes, shapes and forms. Other cell types that not just function as a support unit but also interacts with neurons at the functional level are the glia. Glial cells come in various shapes and forms depending upon their roles and functions, which can range from providng structural stability - to being active players in synaptic plasticity (Tood, et al., 2006). The structural unit through which neuron-neuron, neuron-muscle/organ and glia tripartite relationship becomes a functional unit for all brain functions are termed "synapses". A synapse is a contact point or a hub through which all neuronal communications take place and they most likely outnumber both the glia and the neurons put together. For the sake of this thesis, I will primarily focus on PNS neurons and glia – with specific emphasis on Schwann cells (SCs) and their association with Dorsal Root Ganglia (DRGs) neurons

Similar to neurons, glia also vary in both the CNS and the PNS. In the CNS of some vertebrates, the primary glia population is comprised of oligodendrocytes with the main function

of providing insulation and support to the axons (Nave & Werner, 2014). The primary glia of the PNS on the other hand, is comprised of SCs, and they serve to empower neurons for faster conduction of action potentials across the entire length (Tasaki, 1939). In the PNS, each SC generates one myelin segment which serves to speed up the propagation of action potential particularly for those axons that extend long distance (Tasaki, 1939).

1.2   The Peripheral Nervous System,

The PNS, is involved in the control of a variety of autonomic – sympathetic and parasympathetic systems of the nervous system. Initially, all developing neurons send out processes towards targets located at some distance by invoking interactions between the axons and the substrates (Hay, 1991). Cell to matrix surface interactions generally occur through ligand and receptor interactions such as integrins (Hynes, 1992). Extra cellular matrix (ECM) influences cellular behavior and can regulate cell migration, growth and differentiation (Hynes, 1992). Neurons began their lifecycle like many other cells from mitotic cell division during the development and in most cases they reposition their cell bodies to create the functional nervous system (Flynn, 2013). Neurite outgrowth requires that both axons and dendrites be guided to their proper target by coordination of filamentous actin and the dynamic cytoskeletal polymers that promote shape changes and neuronal polarity. This dynamic cytoskeleton is comprised of microtubules (MTs) (Erik, et al., 2011).

As subsequent neurons emerge, they interact with the pioneering axons through cell-cell interactions that involve a variety of neuronal cell adhesion molecules such as N-Cadherin (Neugebauar, et al., 1988), Fasciculin (Radic, et al., 1994) etc. The structural proteins such as collagen have an important role in creating a scaffold for cell attachment and also tissue and organ

development, and the host response to injury (Badylak, 2002). Functional properties of a scaffold for tissue reconstruction and replacement depend on a variety of factors. These involve the ability to transmit microenvironmental signals to the embedded cells which are served by arginine-glycine-aspartic acid (RGD) peptide that enhance the adhesion of numerous cell types on the scaffold and promote their proliferation and differentiation (Pierschbacher & Ruoslahti, 1984; Yamada & Kennedy, 1984).

Motor and sensory axons in the PNS are thus stuck together by support tissue to create nerves (Kancka & Adameyko, 2014). Individual axons and their corresponding SCs sheathes that are mainly composed of oriented collagen fibers are surrounded by endoneurium. Axons bind together to form nerve fascicles by perineurium, which is built by many layers of flattened cells like fibroblasts and collagen. Finally, an outer sheath of loose fibrocollagenouse - named epineurium binds single nerve fascicles together to create a nerve trunk. Inside the support tissue of nerve the trunk the vessels and veins vascularize the peripheral nerves (Kancka & Adameyko, 2014).

Schwann cells originate from neural crest during embryonic development (Monk, et al., 2015). They turn into SCs precursors between E12-E13 stages. Then during early postnatal phase, immature SCs are created which either have the ability to become myelinating SCs or non-myelin (Remak) SCs (Griffin & Thompson, 2008). Prior to differentiating into a myelin SC, an immature SC passes from promyelin SC stage. This stage is irreversible which means that promyelin and myelin SCs do not convert to immature SCs. Conversion of an immature SC to non-myelin SC is irreversible as well, but myelin SC and non-myelin SCs can be differentiated to repair (Bungner) SCs (Griffin & Thompson, 2008).

Myelinating SC that are surrounded by basal lamina (a layer of extracellular matrix secreted by the epithelial cells), are in close association with the axon and with the overall organization of

the myelinating SC along the length of an axon, nodal, paranodal, juxtaparanodal, and intermodal compartment are created. The nodes of Ranvier is an area of unmyelinated axon, which contains a cluster of voltage-gated sodium channels (Sherman & Brophy, 2005). Higher concentration of Na^+ channels at the nodal region facilitate the propagation of the action potential along the axon and cause conduction of electrical impulses to speed up by jumping from node to node. This phenomenon is called Salutatory conduction (Sherman & Brophy, 2005).

Adjacent to the node of Ranvier (the lateral edge of myelin segment), the cell adhesion molecule contactin, which is associated with glycol protein (CASPR) can be found (Sherman, et al., 2005). Approximately 10-15μm from the paranode, the jaxtaparanode is located adjacent to voltage-gated potassium channels (vgpc's). Adjacent to jaxtaparanode, internode is embedded where axons are wrapped up by tightly compacted myelin basic protein (MBP) (Sherman & Brophy, 2005).

1.3 Cellular and Molecular Signals that Control SC Migration

The number of SCs in the PNS corresponding with axons is regulated by the proliferation and SC precursors apoptosis (D'Antonio, et al., 2006). A variety of factors influence the maintain survival, proliferation and also myelination of peripheral nerves system; among these are TGF-beta and Neuregulin (NRG1) and their respective signaling molecules (D'Antonio, et al., 2006).

Neuregulin-1 (NRG1) is a neuronal growth signaling molecule derived from the largest mammalian gene containing a group of transmembrane and secreting proteins that control the myelination of PNS (Nave & Salzer, 2006). NRG1 signaling is important for glial and neuronal survival, proliferation and differentiation of SCs (Nave & Salzer, 2006). Glia ErbB2 receptors are essential for both SCs expansion and myelination along the axon and also the thickness of myelin

sheath on myelinated axon depends on NRG1 type III expression (Michailov, et al., 2004). Cellular and molecular mechanisms underlying SC precursors migration along nerve fibers remain to be defined but it is known that the SC precursors originate from migrating neural crest cells, move towards a nerve trunk of efferent and afferent fibers and by relocation along these fibers, they are able to en-sheath the neurites (Lai, 2005).

1.4   Regulation of action potential propagation along myelinated fibers

The insulating properties of myelin segments decrease the leakage of Na^+ through the voltage gated Na^+ channels along the axon and facilitate the propagation of action potential (AP) over long distance (Castelfranco & Hartline, 2015). The majority of neurons in the CNS - including cortical and hippocampus are unmyelinated while in the PNS most of the neurons are myelinated. This fact correlates well with the axonal diameter, which in this case is more than 0.2 μm; such axons tend to be myelinated (Waxman & Bennett, 1972). Conduction velocity is linearly proportional to the axonal diameter and myelin sheath thickness, which is classified by their g-ratio (g-ratio is calculated by the ratio between the axonal diameter to the entire diameter of the fiber) (Chomiak & Hu, 2009).

It has been shown that when the g-ratio is between 0.6 and 0.77, the maximal conduction and efficacy is achieved (Pereira, et al., 2012). Furthermore, the g-ratio of a myelinated axon is optimized to obtain the maximal efficacy and physiological function (Chomiak & Hu, 2009). This postulate is validated through observation of the initial hyper-remyelination of demyelinated axons which rebound to the normal g-ratio by increasing the axonal diameter (Blakemore, 1973; Klopfleisch, et al., 2008; Merkler, et al., 2005; Perrot, et al., 2007).

1.5 Multiple Sclerosis (MS) and Demyelination

As mentioned earlier, axonal myelination is necessary to achieve salutatory impulse conduction in the vertebrate nervous system. The multi layered myelin sheath structure is attained by wrapping of the plasma membrane of specialized glia cells and oligodendrocyte in the CNS and SCs in PNS - especially around large-caliber axons. This arrangement is critical, as its perturbations result in neurological diseases such as multiple sclerosis, leukodystrophies and peripheral neuropathies thus giving rise to malformation of the myelin sheath which renders neuronal functions dysfunctional (Jende, et al., 2017).

Cross-talking between axons and glia cells in CNS, and SCs in PNS is crucial for myelin formation, maintenance, re-myelination after injury and for our understanding of disease etiology. Inflammation is a major cause of demyelination and it occurs when the body's immune system attacks the myelin sheath and causes demyelination. According to the national MS society, MS affects 2.3 million people worldwide. White matter in the brain and spinal cord is the location at which the demyelination generally occurs which consequently leads to lesion or plaque formation in the CNS.

1.6 The Involvement of Peripheral Nervous System Demyelination in Multiple Sclerosis

The cellular, molecular and pathological mechanisms underlying MS remain largely unknown. A number of questions that remain unanswered in this field are (Jende, et al., 2017):

- Is inflammatory demyelination of the PNS a part of the same process that affects the CNS in MS?
- Does CNS damage (lesions/atrophy/both) lead to demyelination of peripheral nerves?
- What are the unknown genetic/environmental factors that predispose brain to MS?

Assessing cellular and molecular mechanisms of neuronal myelination are critical to demonstrate how myelination and demyelination processes occur in vertebrate models to understand developmental and neurodegenerative diseases such as multiple sclerosis, however the precise mechanisms remain largely unknown. The issue is difficult to resolve as most cell-cell interactions and the influences/attacking of our immune system of its own organ is often difficult to investigate in the intact animals. Moreover, is it of paramount importance to understand how the process of myelination comes about in the first place. Therefore, most research to decipher fundamental steps involved in neuron-glia interactions has primarily exploited a variety of *in vitro* model system.

1.7 The *in vitro* model system:

All nervous system functions in animals require neuronal assembly during development and the ensuring communications between large networks of neurons which are often difficult to monitor in the intact brain. As such, most labs around the globe have opted to use and explore *in vitro* model systems where neurons are generally grown on two dimensional, plane glass substrates (Kaul, et al., 2004). These two-dimensional substrates have although helped decipher the nutrient needs of various cell types, they do nevertheless limit the ability to decipher fundamental understanding of the mechanisms underlying neuronal growth and synapse specificity. Dr. Syed's lab has defined cellular and molecular mechanisms underlying neurite outgrowth, synapse formation and synaptic plasticity in an invertebrate model where axons are devoid of myelination (Kaul, et al., 2004; Py, et al., 2010; Martinez, et al., 2010; Martina, et al., 2011; Py, et al., 2011; Py, et al., 2012; Luk, et al., 2015; Wijdenes, et al., 2016). In this model, neurons are cultured on the Poly-L-lysine coated substrate which does not mimic the 3-D configuration of neuronal networks that develop and assemble in the intact mammalian brain – thus limiting a direct

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comparison between *in vivo* and *in vitro* conditions. Myelination is however difficult to investigate *in vivo* at the level of single neurons and their corresponding axons. Moreover, there are no suitable *in vitro* models available whereby the process of axon myelination could be studied directly at the level of individual central and peripheral neurons. To achieve myelination, axons needs to be wrapped around by their corresponding glia and this is inconceivable when the nerve fibers are strongly attached to the Poly-L-lysine substrate. In contrast to Poly-L-lysine, collagen on the other hand, offers a 3-D structure in which neurons can be suspended in a 3-D configuration, allowing glia to gain access to axonal membrane to exhibit myelination. However, we still lack a reliable 3-D model where mechanisms of neuronal polarity and myelination could be studied at the level of individual peripheral and central neurons.

1.8   Properties of Hydrogel as Scaffold

Hydrogel networks are synthesized from different types of hydrophilic polymers using crosslinking mechanisms. They exhibit different properties based on how the hydrogel networks are synthesized and the kind of crosslinking method (like physically, chemically or ionically crosslinking) used (Park, et al., 1993). Also each method has its own limitation and advantages depending on the field of use and the application. Because of their exclusive properties like elastic attributes, high percentage of water content and the ability to manipulate these properties, hydrogels are appropriate for various applications. Non-degradable and degradable hydrogels have different usage and applications (Sawhney, et al., 1993; West & Hubbell, 1995; Langer, 1990).

Depending upon the application strategies, various scaffolds have also been used in the field of tissues engineering – e.g. scaffold for bone and cartilage (Hutmacher, 2000), cardiac cell,

vascular cells, muscles and neurons (Hu, et al., 2004). Most of these serve as synthetic, ECM which helps to organize the biological tissue and to grow cells into a three-dimensional (3-D) substrate. The extracellular matrix (ECM) is the substrate of natural tissue that provides structural support to the cell while serving other functions (Nuttelmana, et al., 2002). The synthetic hydrogel must be included in both classical mechanical and physiological parameters (such as biodegradable, porosity and suitable surface chemistry), and biological properties parameters (like biocompatible and cell adhesion) (Nuttelmana, et al., 2002).

1.9   Classification of the Hydrogel

Numerous classifications are considered for hydrogels based on their origin, durability, response to environmental stimuli, and their preparation methods (Gutowska, et al., 1992). According to hydrogel origin, they have been classified into natural, synthetic and semi synthetic (Ullah, et al., 2015). Synthetic hydrogels are synthesized by polymerization of vinyl or vinyl activated monomers. Natural hydrogels usually come from natural polymers including polynucleotides, polypeptides and polysaccharides. Similarly, all natural polymers come from different natural origins. For instance, collagen is taken from mammals and depending on hydrogel stability characteristic in any physiological environment, they can be either durable or biodegradable. Smart hydrogel has been investigated over the past decade (Xia, et al., 2013). However they exhibit unusual changes in their swelling behavior in response to different environmental stimuli like pH, temperature, light, ionic strength and electric field (Xia, et al., 2013).

1.10 Collagen is a Biocompatible Scaffold

A degradable scaffold and bio absorbable polymer matrix serves critical function as an anchor for cell attachment – for it to be growth permissive. It also exhibits other functions like transportability, porosity, binding or releasing biologically active molecule (Perez-Puyana, et al., 2016). Scaffolds that are used for tissue engineering are manufactured from natural materials, which must be similar to that of the organ or tissue where it is to be inserted (Perez-Puyana, et al., 2016). Characteristic properties of synthetic polymers, such as polypropylene or nylon, can be controlled easily and they provide good mechanical stability. Due to their non-degradable nature, they do however pose some risks to immunogenicity in the host tissue (Christensen, 2009). Therefore, the best options are based on proteins including collagen, fibrin and elastin, as these can provide better cell adhesion with least cytotoxicity due to their biocompatible nature (Christensen, 2009). Collagen is one of the most popular molecules in tissue engineering due to its several important physical and biochemical roles in the extracellular matrix of many tissues. Several single cell types, helical polypeptide chains (termed alpha chains), which each contains about 1000 amino acid long comprise this large protein (Perez-Puyana, et al., 2016).

Through a self-assembly process, three alpha chains combine to create a larger rope-like super helix. Further assembling of triple helical molecule into collagen fibrils produces specific binding patterns that are recognizable under electron microscope (Perez-Puyana, et al., 2016). Size of these fibrils is 10-300 nm in diameter and hundreds of microns in length. They can be further assembled into native tissues (MacDonald, et al., 2005). Collagen contains 30% of total protein content and is the most abundant protein in animal tissues. It can be predominantly found in bones, skin, cartilage, tendons and ligaments. Therefore, it has many potential applications, especially in pharmaceutical industry and tissue engineering (Yue, et al., 2015)

1.11 Synthetic 3-D Substrate (GelMA)

One of the most recent 3D environment which has been used widely for different biomedical applications because of its appropriate biological properties and tunable physical features is a gelatin based hydrogel, Gelatin Methacryloyl (GelMA). This synthetic hydrogel resembles essential properties of endogenous ECM (Yue, et al., 2015). GelMA also contains cell attaching and matrix metalloproteinase responsive peptide motifs, which provide a suitable substrate for cell attachment, proliferation and differentiation over GelMA based matrix. GelMA also contains photo-crosslinkers which crosslink by adding photo initiator under UV exposure (Yue, et al., 2015). It can be microfibricated by using various approaches including micromolding, photomasking, bioprinting, and different microfluidic techniques to build substrates with controlled structures. GelMA can also be combined with nanomaterials such as CNT and graphene oxide in order to form network with desired component and to increase the conductive properties and characteristics for specific biological applications (Yue, et al., 2015).

Gelatin - a main component of GelMA contains many argenin-glycin-aspartic acid (RGD) sequences that improve cell attachment (Liu & Chan-Park, 2010). As compared to collagen, GelMA has better solubility and less antigenicity (Maurer, 1954; Gorgieva & Kokol, 2011) which increases the surface contact and for neuronal attachment and axonal generation. The other advantages of using Gelatin in GelMA structure is that a gelatin solution has specific properties at low temperatures to form physically crosslinked hydrogels (Van Den Bulcke, et al., 2000; Djabourov & Papon, 1983).

Furthermore, gelatin can be crosslinked by applying several chemical reactions (Jayakrishnan & Jameela, 1996; Olde Damink, et al., 1995; Sung, et al., 1996; Petite, et al., 1990). Amid groups in gelatin need to be functionalized by methacrylic anhydride to form gelatin

methacryloyl. The functionalized group can then be crosslinked with the assistance of the photo initiator and exposure to light (Yue, et al., 2015). This process is known as photopolymerization and it can take place under mild conditions (room temperature, neutral PH, in aqueous environments, etc). GelMA scaffold provides a 3-D substrate, which promotes temporal and spatial growth of cells (Petite, et al., 1990). Notwithstanding these properties, GelMA has not yet been fully tested on neurons as a 3D scaffold.

OBJECTIVES AND AIMS

The **main objective** of my research therefore was to design/refine a substrate that could act as a scaffold enabling neuronal growth in a 3-D environment. For this purpose, I planned to create a scaffold comprising of nanofibers or nano-layers that could then be coupled with collagen thus compartmentalizing neurons in different layers – in a manner analogous to that seen in the intact brain.

My **general hypothesis** was that GelMa can provide a much better 3-D substrate than both poly D. Lysine and collagen conducive to myelination. My research project was based on two specific aims.

1.11.1 Specific aims:

Aim # 1, (Part A): Designing a 3-D substrate for neuronal growth

In order to mimic the "natural" extracellular matrix, I compared biocompatibility, biodegradability and physiological relevance of two 3D ond 2D substrates (collagen, GelMA and PDL respectively).

Aim# 1, (Part B). Investigating SCs behavior and their interactions with neurons over three types of substrates.

Here I investigated the interactions between DRG neurons and either endogenous SCs or those that were genetically engineered. Neurons were co-cultured with SCs under the above experimental conditions and their potential to interact with neuronal axons was investigated. I sought to determine and define the relationship between SCs and the DRG neurons.

Aim #2: To Determine Whether Myelination Occurs Over GelMA

This aim was focused at determining whether SCs interact with DRG axons and if this interaction would result in the hallmarks of conventional proteins that are associated with myelin. Specifically the evidence for the presence of myelin specific proteins (NF, Nav 1.6, CASPR, MBP and MAG) was sought experimentally by using immunocytochemistry.

2   Chapter 2: Methodology

2.1   Animal Care

Aim #1, To designing a three-dimensional substrate to provide optimum conditions underlying neuronal growth and myelination of Dorsal Root Ganglion Neurons by Schwan cells.

All procedures were performed in accordance with the standards and regulations instituted by the Institutional Animal User Training at University of Calgary, the Canadian Council on Animal Care, and the Animal Use Protocol.

2.2   Dissections

Spinal cords were dissected from Sprague Dawley pups (P0-P3) that were sacrificed by decapitation. DRGs appeared as a cluster of neurons (ganglion) located within the dorsal root of spinal nerve. In order to isolate the DRGs, the spinal cord was removed from the back bone; DRGs were accessible and separated from the trenches of the spines. 15-18 DRGs were isolated from each pup, and for each experiment, 3 pups were scarified. the Dissection and isolating the DRGs have done in dissection solution consisted of Hank's Balanced Salt Solution (Gibco 14185-052), HEPES (Sigma H3376) and the PH and osmolality were adjusted to 7.2 with NaOH and 310 mOsm with sorbitol, respectively.

2.3   Cell Culture

After removing the tissue surroundings including tails and excess tissues, DRGs needed to be treated with penicillin and streptomycin (pen/strep) in L15 media. They were then digested with a digestion solution composed of 1ml F11 DMEM, 4 mg collagenase type I, 40μl Papain (Worthington Labs LS003126), 150mM Calcium Chloride ($CaCl_2$) in 100 mM L-Cysteine

filtered, and 50μl Ethylene diamine tetra acetic acid (EDTA). DRGs were exposed to the digestion solution for 18 minutes, which was the optimal time to achieve sufficient digestion without damaging the DRG neurons and native Schwann cells.

For the next step, the digested neurons were added to the DNase solution (4 μl DNase in 1ml F11 DMEM media). DRGs were then washed with 10% Fetal Bovine Serum (FBS) in F11 DMEM, and then 10% Pen/Strep in L15 media was added again. To dissociate any remaining tissue around the DRGs, the cell solution was triturated in 10% Pen/Strep in L15 media. Then, an empty 15mL tube was coated with 15% Bovin Serum Albumin (BSA) in L15 media prior adding the L15 containing DRGs onto the BSA. BSA consisted of DRGs then was spun at 150g, 800 rotate per minute (RPM) for 6 minutes. The interface and remaining solution were then removed and after washing the cells with 10% L15 Pen/Strep, the DRGs were suspended in DRG media. However, due to the short half-life of Nerve Growth Factor (NGF), the frozen NGF was thawed and used right away before plating the cells.

The density of DRGs were checked under microscope and 300 μl of DRG solution in 5 ml DRG media became ideal for plating. The mixture of DRG and endogenous Schwann cells suspended in DRG media and then 100 μL of cells were placed over three types of substrates including PDL, Collagen and GelMA. After cultivating the neurons, the dishes were incubated for two hours before adding the remaining DRG media (1.9 ml) on each dish. As I mentioned earlier, DRGs derived from spinal cord consist of endogenous Schwann cells which meant that I did not have to add Schwann cells after seeding the cells. Cells were then left to extend their processes in the incubator at 37°C.

2.4 Culture Substrates Preparation

Three types of substrate were prepared to compare the neuronal growth and behavior on 2-D and 3-D substrates. The first environment was Poly-D-Lysine (PDL) which served as a 2-D milieu and also considered as control dish. The second substrate was collagen, which acted as 3D substrate.but due to it could not answer my question about observing neuron – Schwann cells interactions at the level of single axon, I switched to use a semisynthetic gelatin base hydrogel named GelMA with tunable properties that could produce a desirable substrate for my research.

2.4.1 Poly-D-Lysine Application

Glass coverslips were coated with Poly-D-Lysine and Laminin using mixture of 0.65% Laminin [2 µg/mL; Sigma Aldrich], 97.73% Poly-D-Lysine [30 µg/mL; Sigma Aldrich], and 1.62% PBS. 200µL of the mixture was applied to the coverslip of each dish and swirled gently to achieve an even coating. Dishes were incubated at room temperature for one hour. The solution was then removed via aspiration and coverslips rinsed twice with sterile, distilled water to remove excess substrate and to ensure an even layer of coating. Dishes were then allowed to dry for two hours at room temperature before being stored in a fridge at 4°C overnight. The PDL dishes were made a day prior to neuronal culturing.

2.4.2 Collagen Application

All reagents needed to prepared collagen dishes including collagen type 1, NaOH, PBS, H_2O and even the tubes were kept on ice during the collagen dishes preparation. The collagen mixture is created by mixing Collagen type 1 taken from (Sigma Aldrich) (67%), PBS (10%), NaOH (1.7%), H_2O (21.3%). 100µL of this mixture was applied to the coverslip of each dish which was swirled

to achieve an even coating. After collagen application, the dishes were incubated at 37°C with 5% CO_2 in a humidified incubator for approximately two hours, at which point, a firm gel formed. The dishes were prepared fresh on the day of neuronal culturing.

2.4.3 Gelatin Methacryloyl (GelMA) Application

Gelatin Methacryloyl (GelMA) was prepared according to a previously used protocol (Loessner, et al., 2016). Gelatin powder was dissolved in PBS (pH 7.4) in 10% (w/v). For every gram of gelatin, 1 mL of methacrylic anhydride (Aldrich 27668 94%) was added gently over 3 hours while the solution was vigorously stirred at 50⁰C. The methacrylation reaction was quenched by adding pre-warmed PBS at the same volume. The solution was dialyzed against ultrapure distilled water (DW) using a 12-14 Kilo Dalton (KDa) filter for 4 days. After removing impurities and freezing the solution in liquid nitrogen, it was lyophilized in a freeze dryer for 5 days. GelMA hydrogel was prepared by dissolving GelMA macromere in warm PBS containing 0.5% (w/v) photo initiator (Irgacure 2959) at 10% (w/v) concentration. After sterilizing with syringe filter (0.22 μm), the hydrogel was cross-linked with UV light (365 nm, 10mW/cm^2). The stiffness of GelMA hydrogel became tunable within the range of 1 Kilo Pascal (KPa) to 800 kPa by varying the degree of methacryloyl substitution, polymer concentration, photo-initiator concentration, photo-cross-linking time and UV light intensity (Loessner, et al., 2016). In this research, the stiffness of 10% GelMA hydrogel was tuned by using different UV exposure time. The samples were cross-linked under UV for 10, 15, 20, 25, 30, and 35 mins.

After preparing the three types of substrates and purification of cells, DRGs were plated on each dish. Whereas the density of cells in DRG media is an important factor for assessing the neuronal behavior on these substrates, the cell density was tested by adding different amounts of

DRGs (50 µl, 100µl, 150µl, 200µl, 250µl and 350µl) in 5 ml media. However, 350µl was the optimum number of cells in DRG media to see neuronal growth and connection on PDL, collagen and GelMA. After plating 100 µl cells suspended in DRG media, they settled down and attach on the substrates for at least two hours prior to adding the rest of the media (1.9 ml media). 500 µl of the DRG media needed to be changed at least three times a week to remove the debris and refeed the cells by fresh media without FBS to avoid glia cells interference in neuronal outgrowth by over growing. Cells were allowed to extend their neurites in DRG media for three weeks before inducing the myelination.

2.5 Inducing Myelination

Previous studies have shown that myelination in an *in vitro* model can be induced by adding ascorbic acid (AA) in basal lamina to facilitate myelination process by Schwann cells (Rumsey, et al., 2013) For this purpose, 50µg (7%) of ascorbic acid solution was added to DRG media and replaced every other day with 50% of the media.

Cells kept in incubator for three more weeks in the presence of ascorbic acid (AA) may induce myelination. Expression of myelin proteins including myelin basic protein (MBP) and myelin associated glycol proteins (MAG) and also Nodes of Ranvier protein required AA, otherwise SCs did not induce myelin sheath exhibiting myelin functions. Cells could not be kept in incubator for more than 35 days due to contamination and also dissolving of their 3D substrates. Therefore, after 5 weeks in vitro, cells were fixed and stained with specific anti bodies including neurofillament (NF), MBP, MAG, Nav1.6, CASPR and DAPI to assess the myelination in an *in vitro* model.

2.6 Immunohistochemistry

Cultured DRGs and Schwann cells were fixed with 4% paraformaldehyde (Sigma Aldrich) (PFA) in phosphate-buffered saline (PBS) for 10 min, then washed with PBS prior to staining with primary antibodies including chicken anti-αNeurofilament from Novus Biol, mouse anti-voltage gated sodium channel 1.6 (Nav1.6) from UC/Davis NIH Neuromab, Rabbit anti- Contactin Associated Protein (CASPR) from Alomon lab, anti-Myelin Basic Protein (MBP) (from Sigma Aldrich), anti- Myelin Associate Glycoprotein (MAG) (Sigma Aldrich), Secondary antibodies were obtained from Vector Labs (Burlingame, CA, USA).

2.6.1 Myelin Staining

Primary chicken anti-αNeurofilament (NF) 1∘ antibody with different dilution (1:500, 1:350, 1:250) was prepared in fish gelatin to acquire the optimize dilution for fluorescent imaging. Cultured dishes including primary antibodies were incubated at -4° over night.

Secondary antibodies with dilution (1:400) with Alexa Fluor 633-labelled was used to detect neurite outgrowth. Myelin Basic Protein (MBP) and Myelin Associate Glycoprotein (MAG) were detected by using MBP antibody (1:250 dilution), with Alex Flour 488 Labelled as secondary antibody, and MAG antibody (1:350 dilution) with Alex Flour 488 labelled. In order to avoid overlapping the antibodies, in each immunohistochemistry, two primary antibodies were used for each staining. NF and MBP, NF and MAG, NF and Nav1.6, NF and CASPR. NF was used in every immunostaining to detect single neurite emerged from somata and track the neurite-SC interactions.

2.6.2 Nodes of Ranvier Staining

Nav 1.6 (Voltage Gated Sodium Channel 1.6) and CASPR (Contactin Associated Protein) are located in Nodes of Ranvier in lower density as such higher concentrations of antibody was needed. Due to the fragile structural integrity of 3D substrate used in my study, the samples could not be incubated for more than 5 weeks - the time required to visualize the Nodes of Ranvier's proteins. Nav 1.6 and CASPR antibodies were used to detect.

2.7 Electroporation

Electroporation or electro-permeabilization is a biological technique used to transfect cells like SCs. In this technique, an electrical field is applied to the cell to enhance membrane permeability which allows chemicals, drugs or DNA to diffuse into the cells. To visualize the SCs behavior, a cell line of SCs tagged with GFAP was introduced via electroporation. Three different concentrations were prepared, 200 μL, 300 μL and 400 μL to acquire the optimal transfection. By monitoring cell growth for 7days, I found that the 300μL concentration was ideal for cell growth and for observing cell behavior via microscopy.

2.8 Scanning Electron Microscopy (SEM) Scaffold Characterization

Scanning Electron Microscopy (SEM) was used to reveal the scaffold microstructure and also to compare the porosity and available contact surface of the Collagen and GelMA substrates. Two types of samples were prepared; GelMA and Collagen without neurons, and also both substrates with seeded neurons to illustrate the differences in neuronal behavior and outgrowth between the two conditions.

Samples were washed in phosphate-buffered saline (PBS) and then snap-frozen using liquid nitrogen for 5 minutes. After freeze-drying overnight, the samples were sputter-coated with gold and finally imaged using a scanning electron microscope (Philips XL30).

2.9   Measuring Mechanical Properties

Compression strength was evaluated using a ElectroForce 3220 Dynamic Mechanical Analysis (Bose, Inc.). Disked shaped GelMA samples were prepared at different UV exposure time ranging from 10 to 35 mins. Before loading under compression, the hydrogel samples were incubated in PBS at 37°C for 2 hrs. The applied strain percentage was under control from 0% to 40%. The linear part of the slope of stress–strain curve (from 0% to 10% strain) was used to estimate Young's modulus.

3   Chapter three

Neuronal Survivability Results

Aim #1 (Part A): Designing 3-D substrate for neuronal growth

Aim# 1, (Part B). Investigating the SCs behavior and their interactions with neurons over three types of substrates.

3.1   Dorsal Root Ganglia Neurons Isolation, Survival and Outgrowth *in vitro*

To establish a model system whereby *in vitro* isolated, Dorsal Root Ganglia Neurons (DRG) could be cultured on substrates that supported neuronal survival and foster neurite outgrowth, I began first with an already established protocol employing Poly-D-Lysine (PDL). Specifically, I sought to determine, whether isolated DRG neurons when plated on PDL (used as a two-dimension (2D) substrate), would survive and extend neurites. DRG neurons were isolated from the intact ganglia on Day-1 and plated on Laminin coated dishes (PDL). The isolated cells when examined on Day-2 not only survived (85% - n = 130 Figure 3:1) but also exhibited processes that were 300 μM in length. Figure 3:2A Shows the cultured neurons on Day 2 and Day 15 respectively (Figure 3:2B). On PDL cells were generally maintained for 5 weeks in incubator but they acquired the optimal growth by day 15. These experiments provided a standard benchmark of neuronal survivability and growth and were thus used to compare and contrast with all subsequent substrates.

I next sought to determine the interactions between the DRG neuronal processes and the native Schwann cells (SC) that often accompanied the cell bodies during the isolation and dissociation procedure.

Whereas, I did observe SC often interacting with the axon (adhere or crawl along the neurite – Figure 3:3), neither did I see a single SC "wrapping" around the neurite, nor their

clustering/bundling was apparent along the length of the axon. I attributed this inability of SC to axonal wrapping to direct and strong adhesion of DRG axons to PDL thus limiting accessibility to axons.

To determine if a 3D substrate could provide scaffold for neuronal outgrowth – collagen, a natural hydrogel was next used. For this purpose and to examine the survivability and the extent of neurite outgrowth of DRG neurons on a collagen substrate, neurons were isolated as above, and examined on day 1 to day 35 (5 weeks in culture). I found that both the survival rate of neurons cultured on Collagen (approximately 60%, n=90 see Figure 3:1), and the extent of their neurite outgrowth was significantly less than that of the PLD (150μM, Figure 3:4). Moreover, I observed that unlike PDL, the cultured neurons did not interact freely with SC, rather they appeared to cluster and bundle around the axonal processes. Under these culture conditions however, I noted that a multitude of axonal processes emanated from the cell body and often bundled into larger clumps thus making it difficult to visualize - one axon to single SC interactions (Figure 3:5). These culture conditions thus made it difficult to decipher clear interactions between the SC and the axons.

Taken together, the above data demonstrate that whereas both PDL and Collagen promote neuronal survival and growth they do not however appear suitable for studies involving myelination.

Next, I sought to search for a 3-D substrate which would be more *in vivo*-like, then I came across Gelatin Methacryloyl (GelMA) which is a gelatin base synthetic hydrogel with modifiable mechanical properties that could produce the designer 3D substrate with the ability to prevent axonal bundling in a manner analogous to that of *in vivo*.

By varying the degree of polymer and photo-initiator concentrations, photo-crosslinking time and UV light intensity, the optimized condition for neuronal growth was achieved.

3.2 Fundamentals of Designing a Biocompatible, 3-D Scaffold (GelMA) for Neuronal Growth.

3.2.1 Necessity of Functional Groups for Developing a 3-D Substrate

Gelatin, which contains OH (Hydroxyl) and NH_2 (Amid) groups is the basic material for creating a porous environment. These groups however are needed to be functionalized with Methacrylic Anhydride (MAA) and to create functional groups (Figure 3:6). The gelatin with functional groups called GelMA precursor, binds together and creates a 3D substrate after exposure to photo initiator under UV light. The resulting microfibers then act as scaffold for neuronal growth. Two concentrations (5% and 10%) of Gelatin were used to determine which one would create a better biocompatible substrate. The first factor that I assessed was meant to obtain optimum structure with the highest biocompatibility properties as measure by the photo crosslinking time.

3.2.2 Optimum Photo Crosslinking Time

As mentioned earlier, functional groups on GelMA precursor solution needed to bind together after exposure to photo initiator under UV light in order to create a 3D substrate. Photo crosslinking time was an important factor that allowed us to observe the optimum scaffold behaviour of the substrate. A range of curing time was then tested (1, 5, 10, 20, 25, 30, 35, 40, 45 minutes) to determine the best cell attachment and survivability on this substrate.

By running different experiments under same situation, in collaboration with Biomedical Engineer (BME) Department, Dr. Amir Sanati Nezhad's lab and the generous expertise of Mohsen Janmaleki, Ph.D. student in Amir's lab, eventually, 25 minutes became the optimum photo

crosslinking time that turned GelMA into a template for cell attachment and rendered it bio compatible. The resulting extracellular matrix then served to provide structural support for neuronal growth (Figure 3:7). Considerable efforts and iterative process was followed to obtain the optimum crosslinking time thus allowing me to understand the scaffold configuration and the porosity structure of GelMA. To define the structural integrity of GelMA, Scanning Electron Microscopy (SEM) techniques were next employed.

3.2.3   Scanning Electron Microscopy (SEM)

In order to confirm that GelMA indeed provided porosity and created 3-D configuration conducive to neuronal growth, SEM images were acquired. Figure 3:8A shows GelMA structure with 5% gelatin concentration, and Figure 3:8B revels GelMA configuration with 10% gelatin concentration. For both of these instances, the crosslinking time under UV light 25 minutes was found to be the optimum crosslinking time. Less gelatin concentration revealed larger pore diameter and the functional groups that were crosslinked under UV light as depicted in the Figure 3:8A. As expected, Higher gelatin concentration (10%) showed smaller pore diameter with more contact surface for neuronal attachment.

The SEM photographs indicated that GelMA with 5% gelatin concentration exhibited less contact surface and larger porous diameter, which I deemed to be unsuitable for neuronal cell attachment, growth and neurite extension. For *in vitro* studies, in addition to cell attachment properties, scaffolds must be considered as a delivery vehicle for nerve growth factor/s required for neuronal outgrowth. *Figure 3:8*C shows the SEM photograph of collagen surface, which is similar to GelMA with 5% gelatin. Both 3D surfaces produce high porosity with less contact surface for neuronal growth.

Figure 3:9 shows the SEM photographs of GelMA surface and deeper layer in lower magnification. Figure 3:9 A, B indicate the cross-over porosity inside the GelMA and reveals less condense pores with larger diameter in comparison with surface. Figure 3:9C shows a cell body cultured on GelMa at day 3; the neuron exhibited sprouting and extended processes projecting through the deeper layers of GelMA.

3.2.4   Measuring the Mechanical Properties of GelMA

Our primarily data lend considerable support for the hypothesis that GelMA may serve as a scaffold structure that could provide anchor for neurite suspension. However, 3-D scaffold properties are also contingent upon several other features of the biomaterial that was applied to the 3D structure. The possible advantages of 3D engineered constructs could be revealed and confirmed further by direct experimentation. Thus, similarities in tissue engineered stiffness and mechanical properties created by this environment for neuronal growth, is one of the most important factors that mimics an *in vivo* extra cellular matrix.

Based on the above criteria, creating a 3D substrate that represents the same stiffness and strength as observed in an intact tissue was deemed essential for *in vitro* studies. The mechanical properties measured (as mentioned in the methods and material section) are presented in Figure 3:11. Specifically, the stiffness of the hydrogels was measured by ElectroForce 3220 Dynamic Mechanical Analysis (Bose, Inc.) for different UV exposure time. Unconfined compression was applied on samples with different curing time of 10, 15, 20, 25, 30, and 35 minutes. In general, increasing curing time enhanced the stiffness for 10% GelMA hydrogel as shown in the representative curve in

Figure 3:10. The compressive moduli were estimated based on the linear part of the slope of stress–strain curves which is illustrated for a sample with 25 minutes of curing. Figure 3:11. shows that the mechanical properties of the hydrogel have a direct correleation with curing or UV exposure time; the compressive modulus increased by three times from about 15 kPa to 45 kPa when the curing time increased from 10 minutes to 35 minutes.

3.3 Biocompatibility of GelMA

Unlike collagen, the GelMA hydrogel was freshly prepared immediately before neuronal seeding and extensively washed out with PBS to separate the residue of photoinitiator that might have toxic effects on neuronal growth. When cultured on GelMA, the neurons exhibited 65% survivability (n=100 - see Figure 3:1), and the length of their neurite outgrowth was 400 μM - longer than both collagen and PDL. Figure 3:12 reveals the sprouting response of the soma (Figure 3:12A) and neurite outgrowth extension (Figure 3:12B) on GelMA. Like PDL and collagen, GelMA's ability to extend neurite outgrowth was investigated further to determine if this novel 3-D substrate indeed produced single axons, which were suitable for SCs interactions and wrapping. For this purpose, neurons grown on GelMA were stained with neurofillament (marker of axon) and individual axons contacted by SC were revealed by immunocytochemistry (Figure 3:13).

3.4 A comparative account of Neuronal Survival Over Three Types of Substrates

To compare differences between three types of substrates including PDL as a 2D and collagen and GelMA as 3D substrates, a variety of factors were investigated. First, the ability of seeded neurons to exhibit sprouting was assessed. Figure 3:14 shows cell body of a sprouted neuron on PDL,

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collagen and GelMA and illustrates the differences between these three kinds of substrates. Soma cultured over collagen looked bulkier and rounded, and extended sprouts in one direction more than the others substrates tested.

The processes also extended in different focal planes demonstrating that the axons were traversing through different layers of a 3D substrate. The neuronal cell body cultured on GelMA appeared to be of flattened shape and its sprouts spread in different directions. GelMA promoted better survivability and sprouting ability compared to collagen, which was similar to that of PDL. After 48 hours in culture, cells began to sprout and extended neurites at different growth rates - irrespective of the neuronal density.

3.5   comparative account of biocompatibility and survival on three substrates.

Next, I sought to compare and contrast the ability of three different substrates in the context of their biocompatibility. Neurons began to sprout 24 hours after platting on PDL and 48 hours on collagen and GelMA. This serves to suggest that PDL promoted growth at a faster rate than the other two substrates. However, not all neurons that survived exhibited outgrowth. The extent of total growth was measured to determine differential growth promoting attributes of all three 3 substrates. Figure 3:15 shows different morphological aspects of axon outgrowth on PDL and collagen and GelMA. On PDL, neurons attached to the bottom of the culture dish and were although surrounded by SCs they did not interact freely with them (Figure 3:15A). Figure 3:15 B shows neuronal grown on collagen whereby growth resulted in fasciculated axons bundles that emanated directly from the somata. Such an aberrant growth pattern often made it difficult to decipher one-on-one axon to SC interactions.   In contrast, when GelMA was used as a

biocompatible hydrogel, neuronal sprouting that occurred from the somata was in the form of single axons.

Figure 3:16 shows the percentage of neurons exhibiting outgrowth under different experimental conditions. I noted that 75% of neurons that survived on PDL exhibited neurite outgrowth (n=90), 50% (n= 45) over collagen and 65% (n= 60) on GelMA, which meant that GelMA served as better 3D substrate compared to collagen whereby neuronal survival rate was higher.

3.6   A comparative account of Neurite Outgrowth on three substrates

I next sought to determine which substrate was the most growth permissive by measuring the extent of total neuronal growth. Neurons were cultured on PDL as controls and on collagen as a 3D substrate. As expected, neurons extended processes on PDL at a much faster rate and the extent of total growth was greater than collagen (Figure 3:17A).

On PDL, 70%, (n=95) of neurons extended their neurite, whereas on collagen, the of neurons extending processes dropped to around 28% (n= 15 out of 45 neurons). The rest of neurons either stopped extending neurite after the initial outgrowth or did not survive after a few days. GelMA on the other hand, promoted neurite extension from about 50% (n= 35 -

Figure 3:18).

Comparing the neuronal extension appearance on PDL, collagen and GelMA substrates revealed that GelMA and collagen acted as 3-D scaffold for neuronal growth. Tracking the focus on the cell bodies in Figure 3:17B and Figure 3:17C ,the neurites outgrowth, the image drops out of the focus suggesting that the axons were traversing through the substrate freely and into various different layers.

3.7   Comparing the length of Neurites on Three Types of Substrates

By measuring the total length of outgrowth in all three dimensions with florescent z-stacks, I learned that neurons grown on PDL exhibited growth within a range of 300 μm neurite length. Growth on both collagen and GelMA however exhibited different behaviours. Specifically, neurite length on GelMA spanned the area > 400 μm (median = 280 μm, maximum = 430μm) which was longer than both PDL (300μm) and collagen (180 μm – see Figure 3:19).

3.8   Effect of substrate on neuronal connectivity

Comparing the neuronal connectivity on PDL, collagen and GelMA was next assessed to determine which environment served as the best substrate for neuronal adhesion.

Dissociated rat neonatal DRG neurons were seeded on PDL, collagen and GelMA and cultured for up to 5 weeks to measure how neuronal connectivity can be formed over these three substrates. PDL is a rigid substrate with the highest stiffness, which leads to highest neuronal connectivity in 2D configuration. Collagen as a 3D substrate developed a network of neuronal bundles with fascicles of axons, which is not ideal for monitoring myelination or any neuron - SC interactions.

Figure 3:20 shows axonal cluster at 15 days *in vitro* over GelMA. Compared to Collagen and PDL, a higher density of neurons needed to be initially plated to produce a stronger axonal bundling. This was established by testing different dilution of DRGs in F11 demem media, and resulted in the observation that the higher density of cultured neurons leads to the formation of a

stronger network. Taken together, these studies demonstrate that by comparing the survivability of DRG neurons on PDL as 2D substrate and collagen and GelMA as 3-D scaffold, I concluded that Gelatin base hydrogel (GelMA) developed a 3-D substrate for neuronal growth, and neurite extension. It also provided a conductive environment for neuronal connection. Tunable chemical and mechanical properties of GelMa helped me to manipulate different factors such as the gelatin concentration and photo-crosslinking time, design a willing substrate to peruse my studies further.
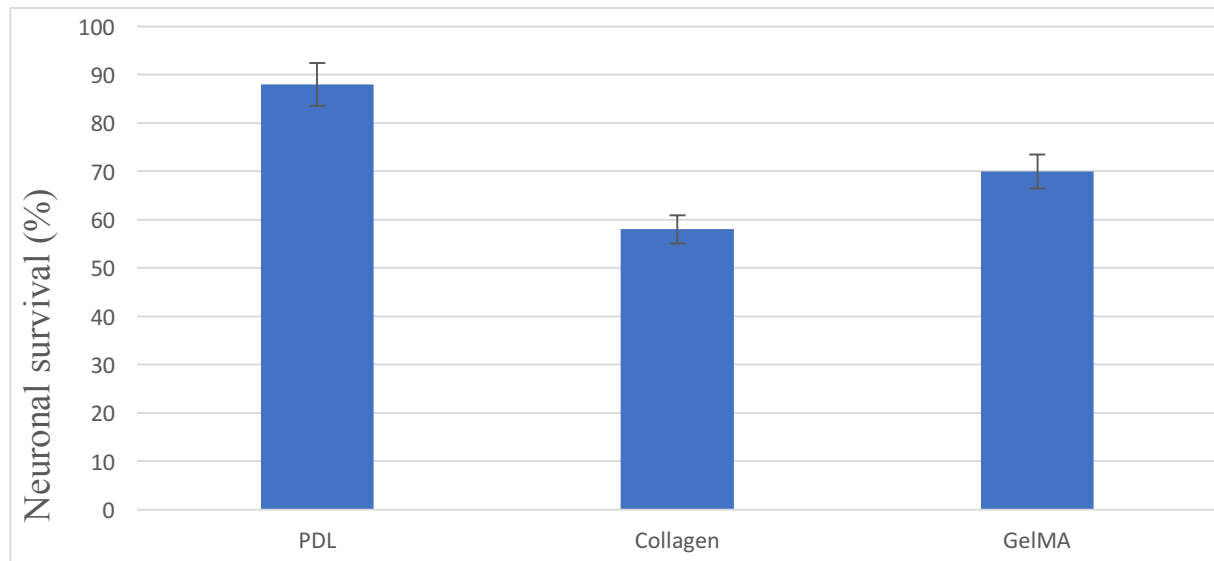
Figure 3:1 The percentage of neurons that survive over PDL, Collagen and GelMA. 80% of neurons on PDL survived 24 hours after culturing, 55% on collagen and 65% on GelMA.
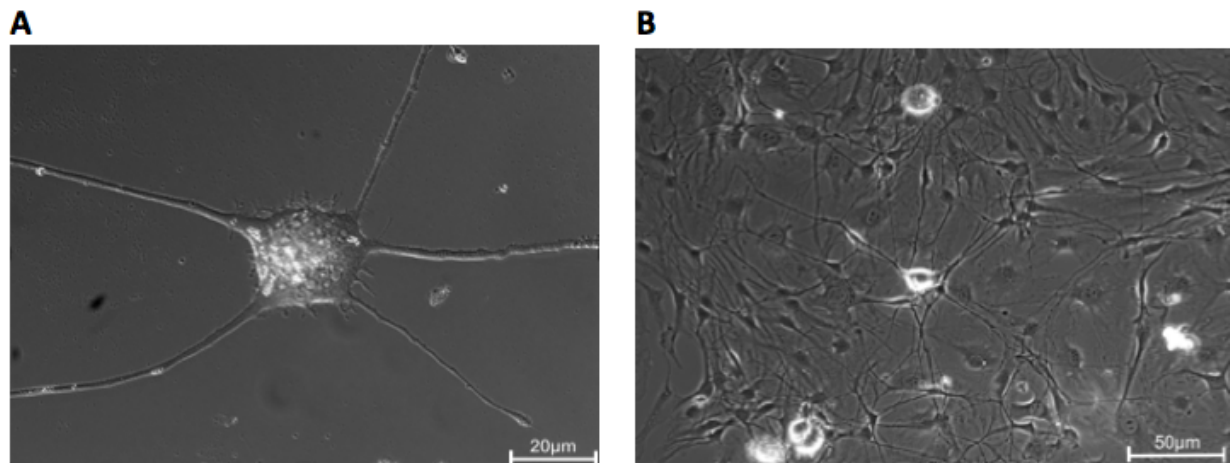
Figure 3:2 Survivability and the ability of neurons to sprout and extend neurite on PDL. (A) Cultured neurons on PDL on Day 2, (85%, n = 130) survived and sprouted well. (B) (75%, n=95) of survived neurons exhibited neurite outgrowth on Day 15.
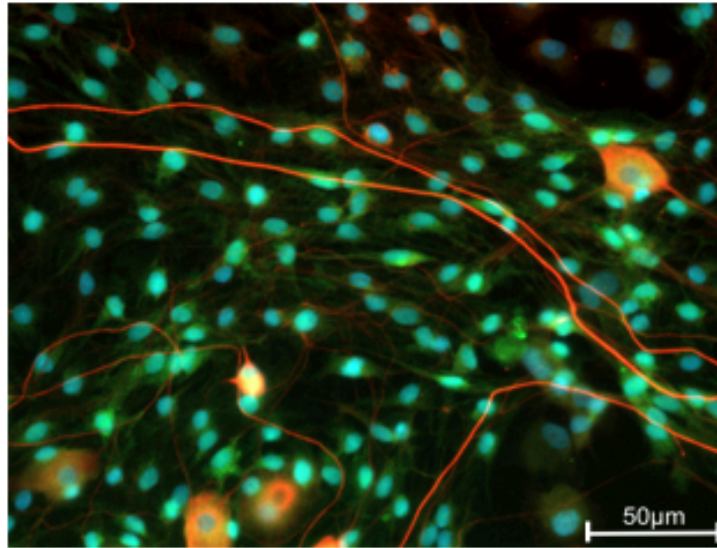
Figure 3:3 SCs that accompanied the DRGs interact with sprouted axons without any sign of enveloping the axons. Axons stained with neurofillament (red color), green color shows the overlapping of the red color with DAPI which represent the nuclei of SCs. Inability of SCs to wrap around the axons suggests the strong adhesion of DRG axons to PDL.
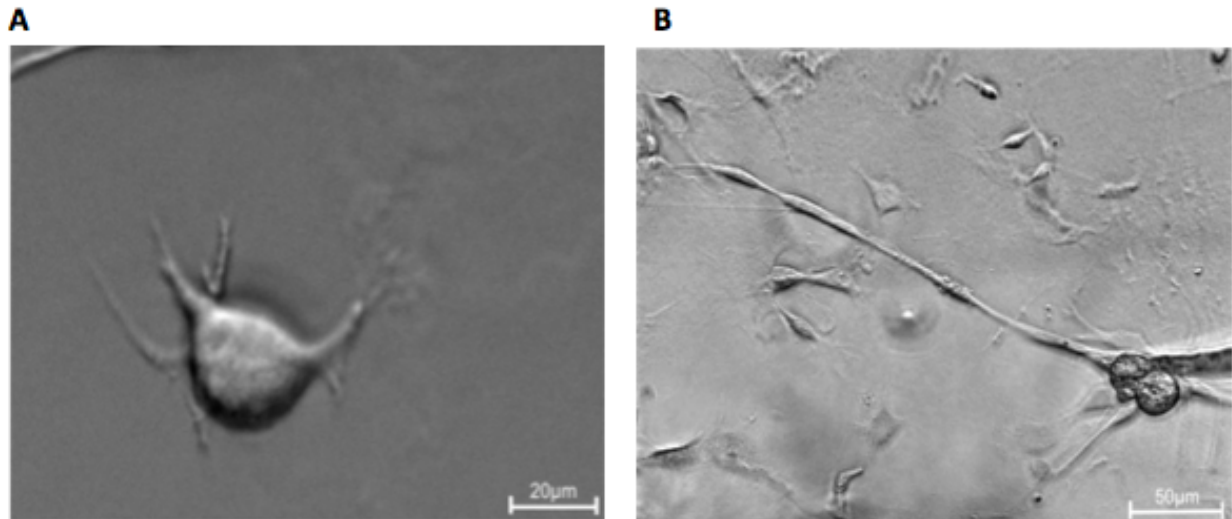
Figure 3:4 (A) Morphological attributes of sprouted neuron on collagen on Day 2 (60% (n = 90) neurons survived and sprouted in spherical shape, (B) (50% (n=40) neurons exhibited neurite outgrowth on Day 15.

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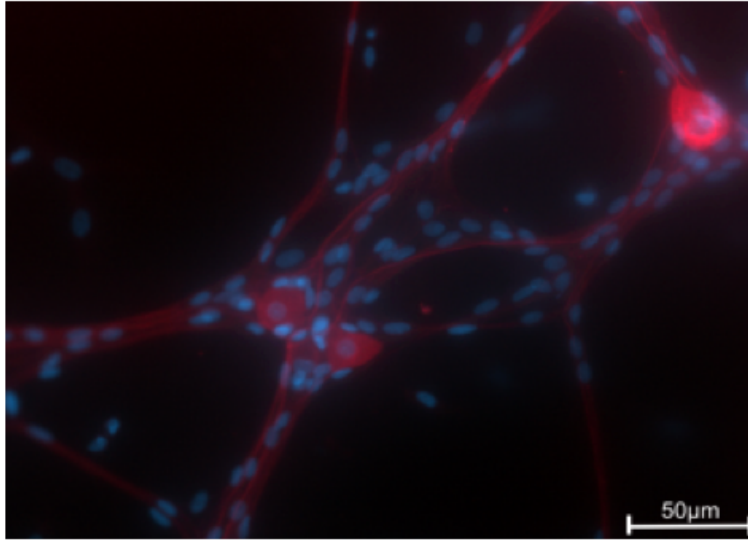
Figure 3:5 Fascicle of neurite emerged from the somata when neurons are cultured on a collagen substrate. Co-cultured SCs seeded with neurons on collagen did not freely move around the neurite. Blue color, which is nuclei of the SCs demonstrates that SCs that are in contact with axons (red color) live longer than the ones that are not in contact with neurites.
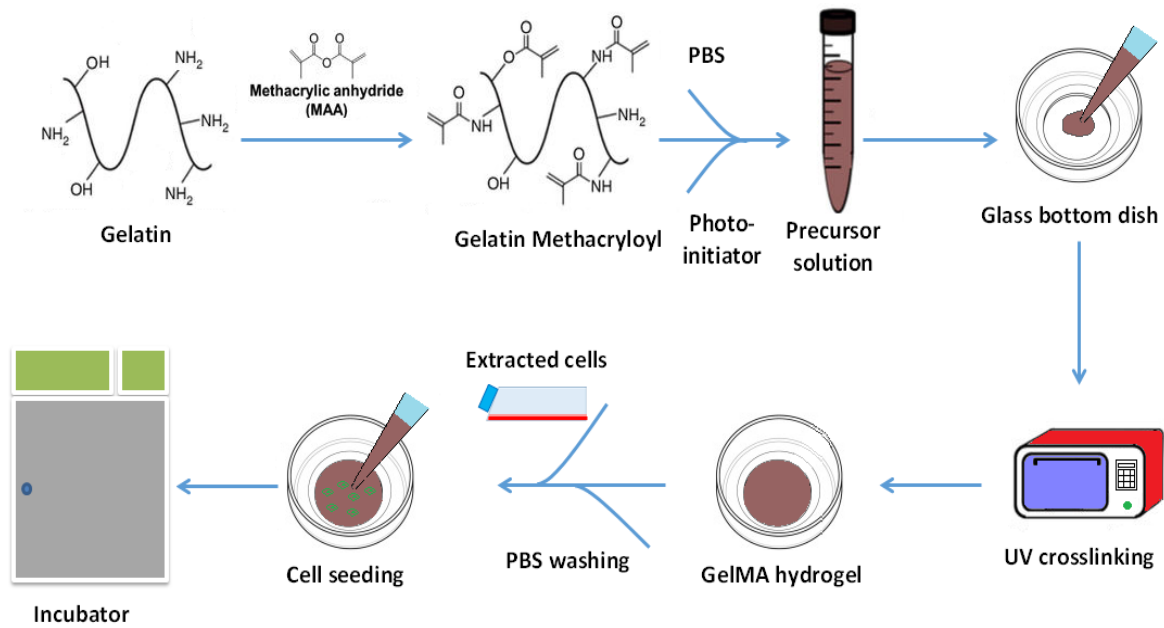
Figure 3:6 Schematic showing the GelMA preparation, purification and polymerization under UV light. OH (hydroxyl) and NH_2 (Amid) groups on gelatin functionalized with Methacrylyc anhydride (MAA) and it turned to precursor solution in presence of photo initiator. The culture dishes were coated with precursor solution and hydrogel was polymerized under UV light. The GelMA dishes needed to be prepared freshly on the culturing day.
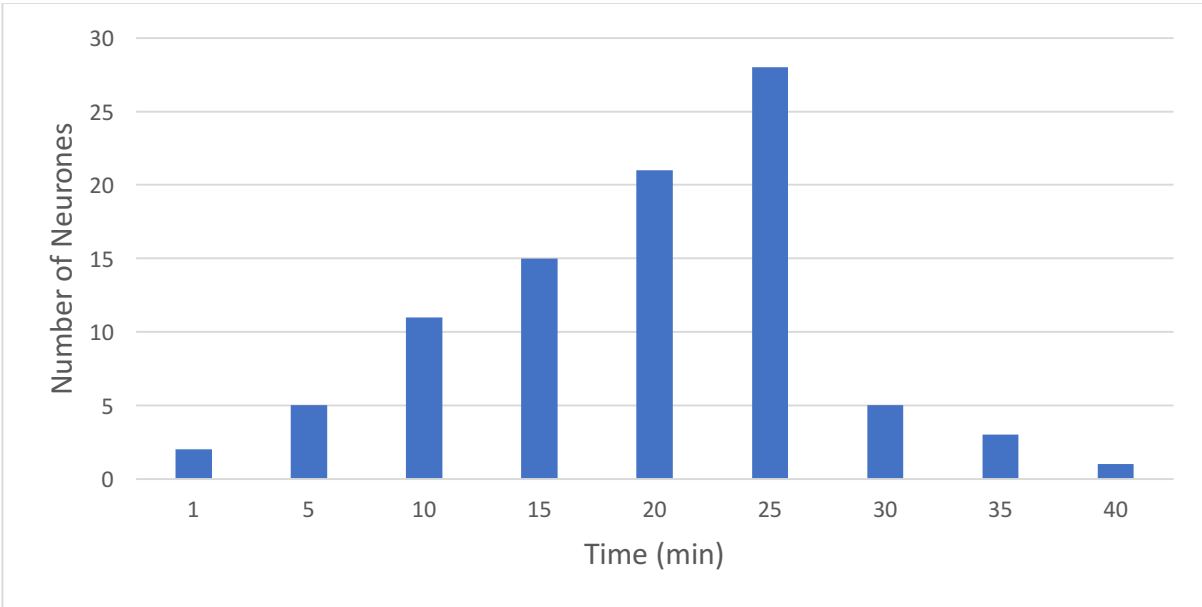
Figure 3:7 Bar graph showing the photo crosslinking time (1, 5 10, 15, 20, 25, 30, 35 and 40 minutes) for developing the GelMA. The optimum crosslinking time for cell attachment and forming a biocompatible extracellular matrix (ECM) on GelMA shows that 25 min exposure yields the best results in the contexts of neuronal survival
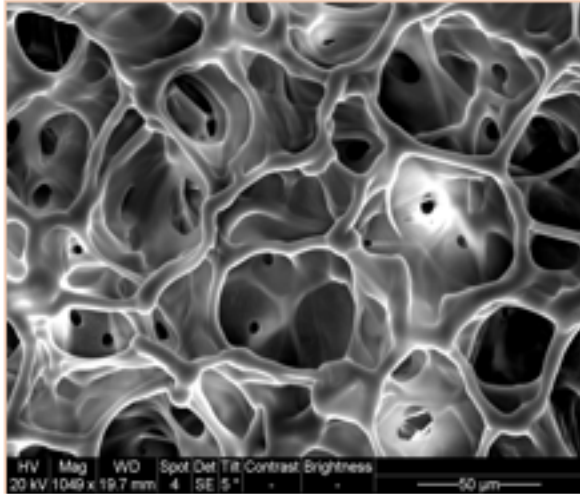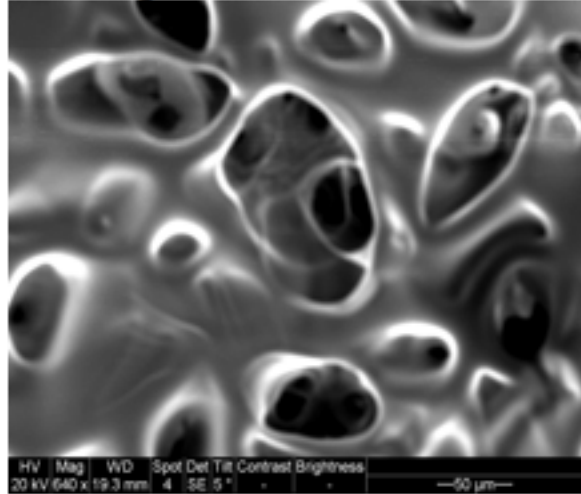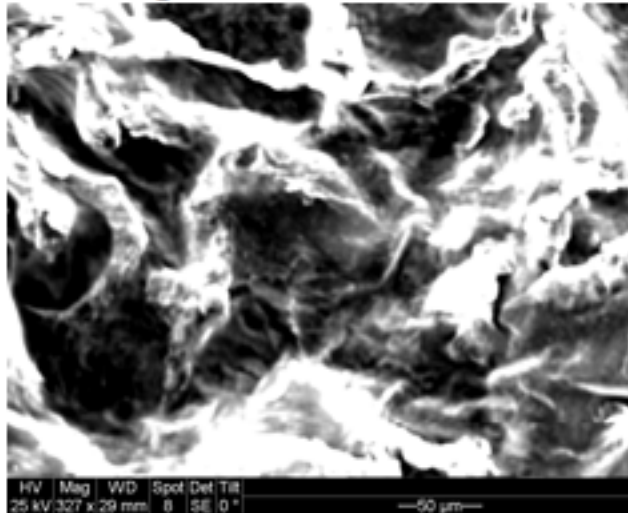
Figure 3:8 SEM photographs show the surface porosity of GelMA on different gelatin concentration and collagen. (A) GelMA with 5% gelatin concentration vs (B) GelMA with 10% gelatin concentration. A) Shows larger pore diameter with less contact surface for cell adhesion. B) Indicates higher contact surface with smaller pore diameter for neuronal attachment. C) collagen surface. Collagen indicates similar porosity as 5% gelatin.
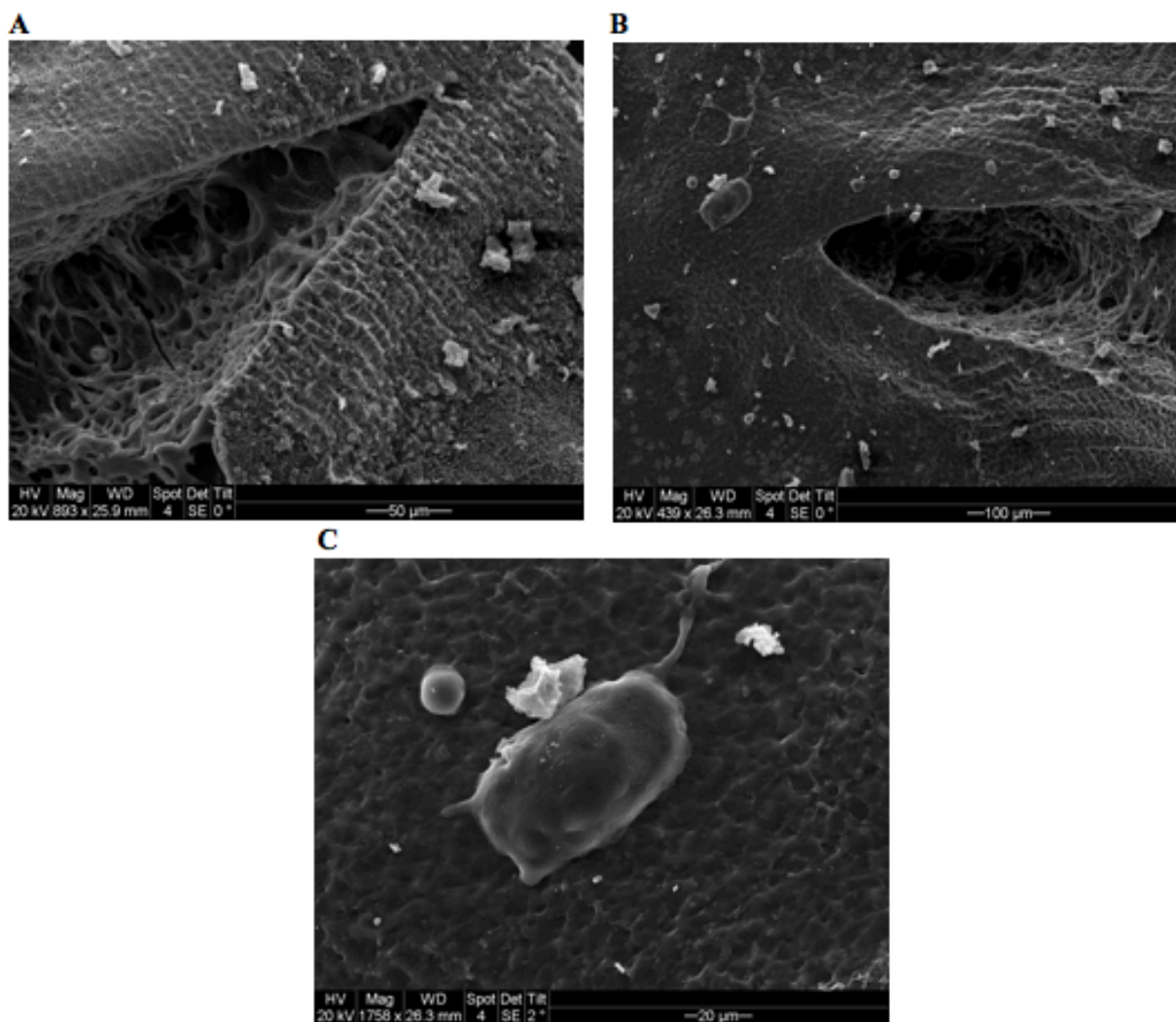
Figure 3:9 Crossover structure of GelMA. A and B exhibit the inside porosity of the GelMA at different focal planes. C) shows the cell body cultured on GelMa at day 3, and showing the neurites were sprouted through deeper layer of GelMA. The figure shows that GelMA could develop as a 3D substrate allowing neurons to extend neurite into different focal planes in deeper layers.
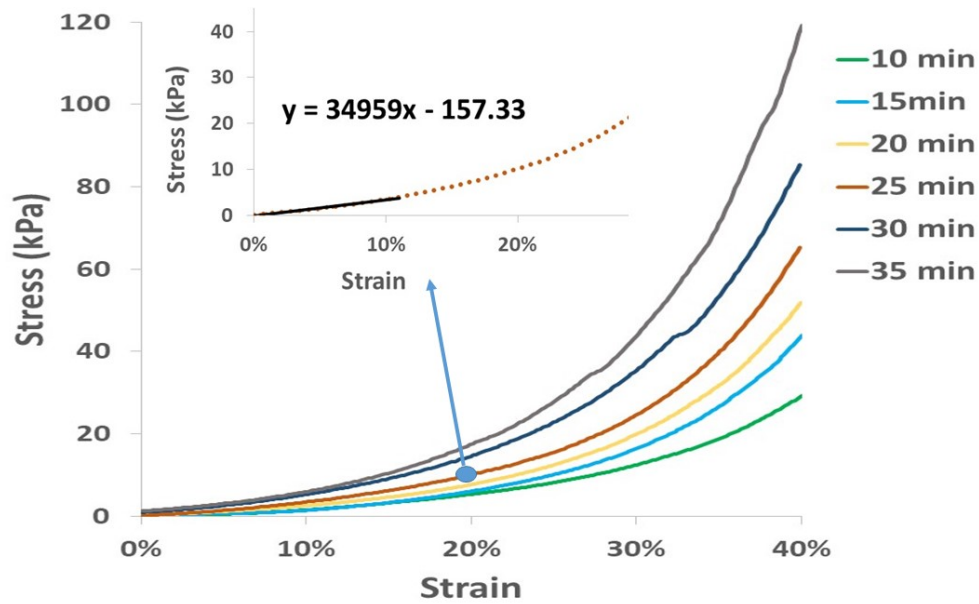
Figure 3:10 Mechanical properties of 10% GelMA for different UV exposure times was measured and the stress and strain curves for comparison tests are obtained. Elastic moduli were estimated from the slope in the linear region of the curves and the stiffness of GelMA acquired 34.95KPa which is similar to human tissue for cell growth.
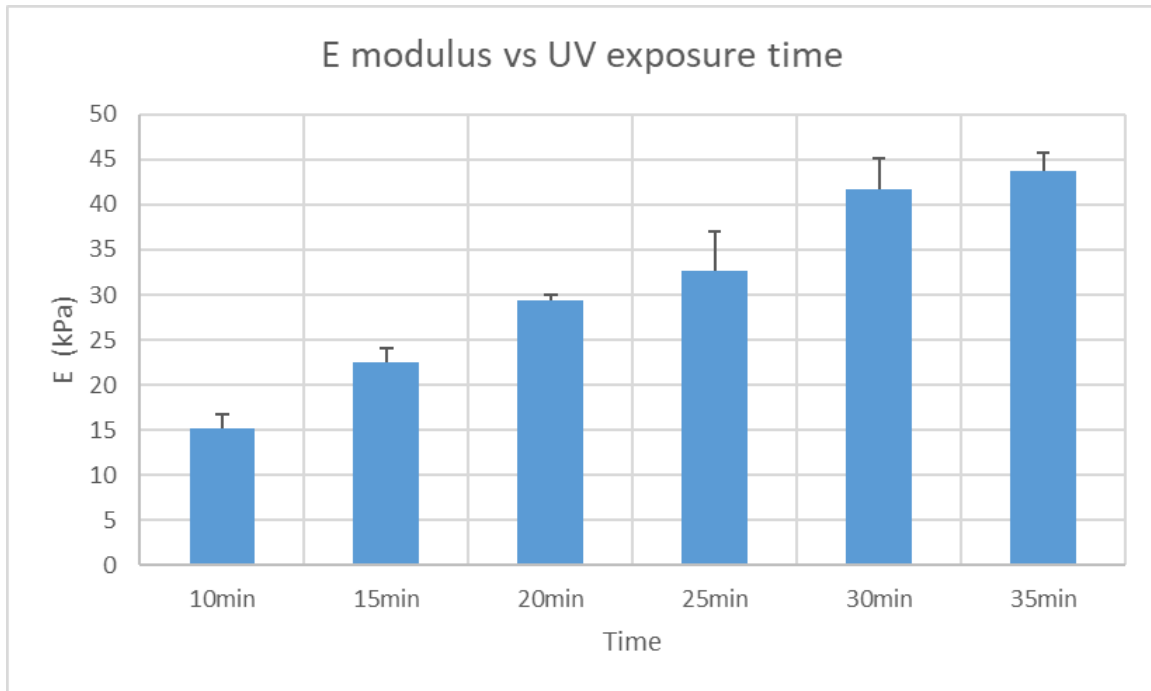
Figure 3:11. Compressive moduli were measured after curing GelMA samples for specific exposure duration and the module for 25 minutes curing time obtained 34.95 KPa which yielded the most biocompatible properties for neuronal outgrowth. This figure shows that the stiffness of GelMA is 35 KPa which is the moderate stiffness for neuronal outgrowth of human tissue in a tissue.
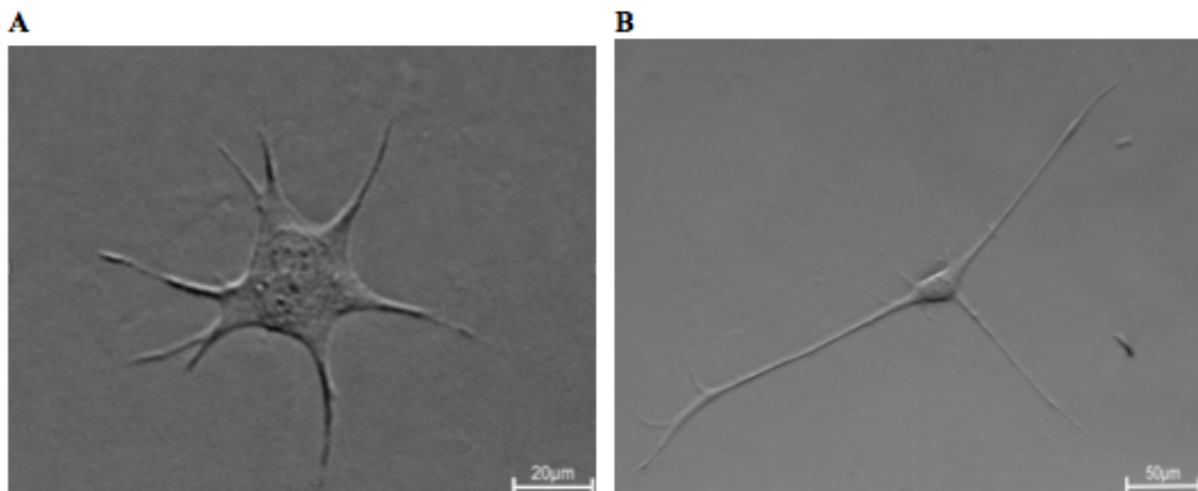
Figure 3:12 Survivability and the ability of neurons to sprout and extend their neurite on GelMA. (A) shows a sprouted cell body on GelMA day 2 (n=100 sprouted in flattened shape), (B) 65% (n=65) of neurons exhibited neurite outgrowth on Day 15. The neurite branched and extended filopodia which is more obvious than that of PDL and collagen.
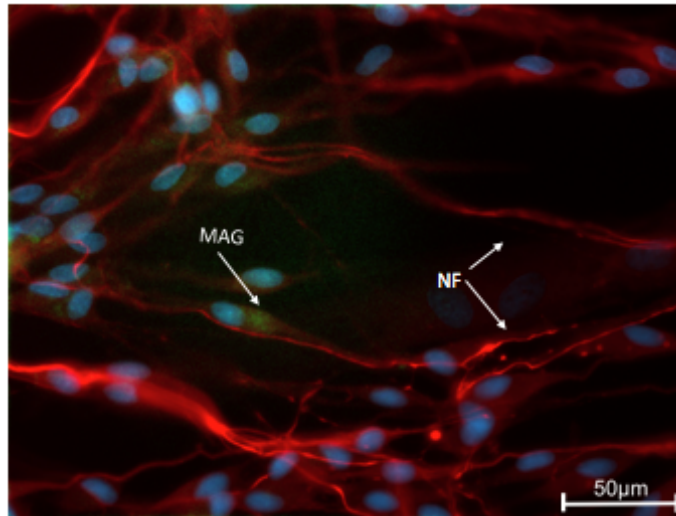
Figure 3:13 Neuronal outgrowth on GelMA shows interaction between axons and the SCs. White arrows show the mature SCs (green color, stained with MAG) interacting with axons (red color), stained with Neurofilament the marker of axons
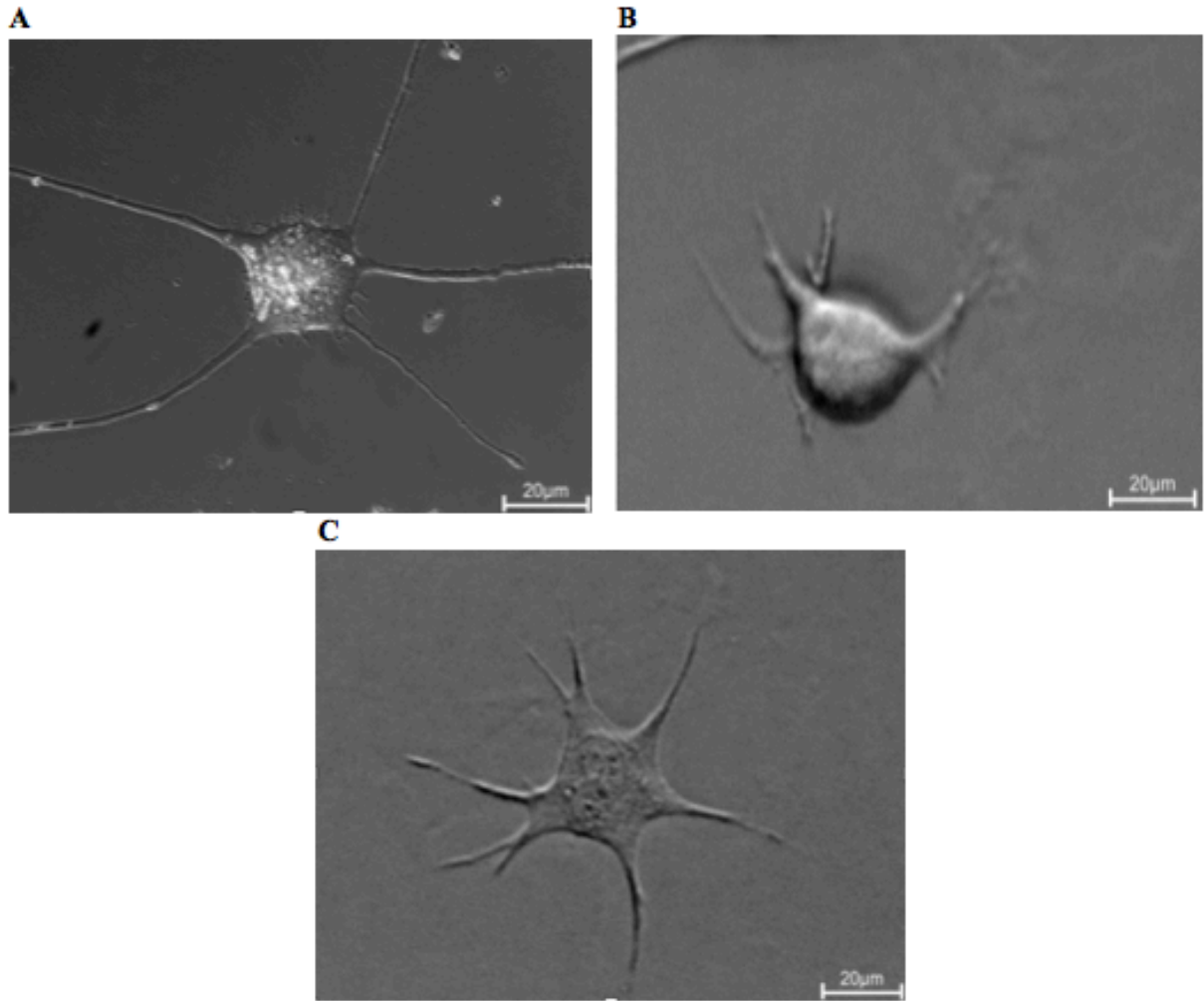
Figure 3:14 Comparison of sprouted neurons on A), PDL B), collagen C), GelMA (A). Somata cultured over collagen (B) look bulkier and rounded shape, while PDL (A) and GelMA (C) show flattened shape and their neurites spread in all directions.
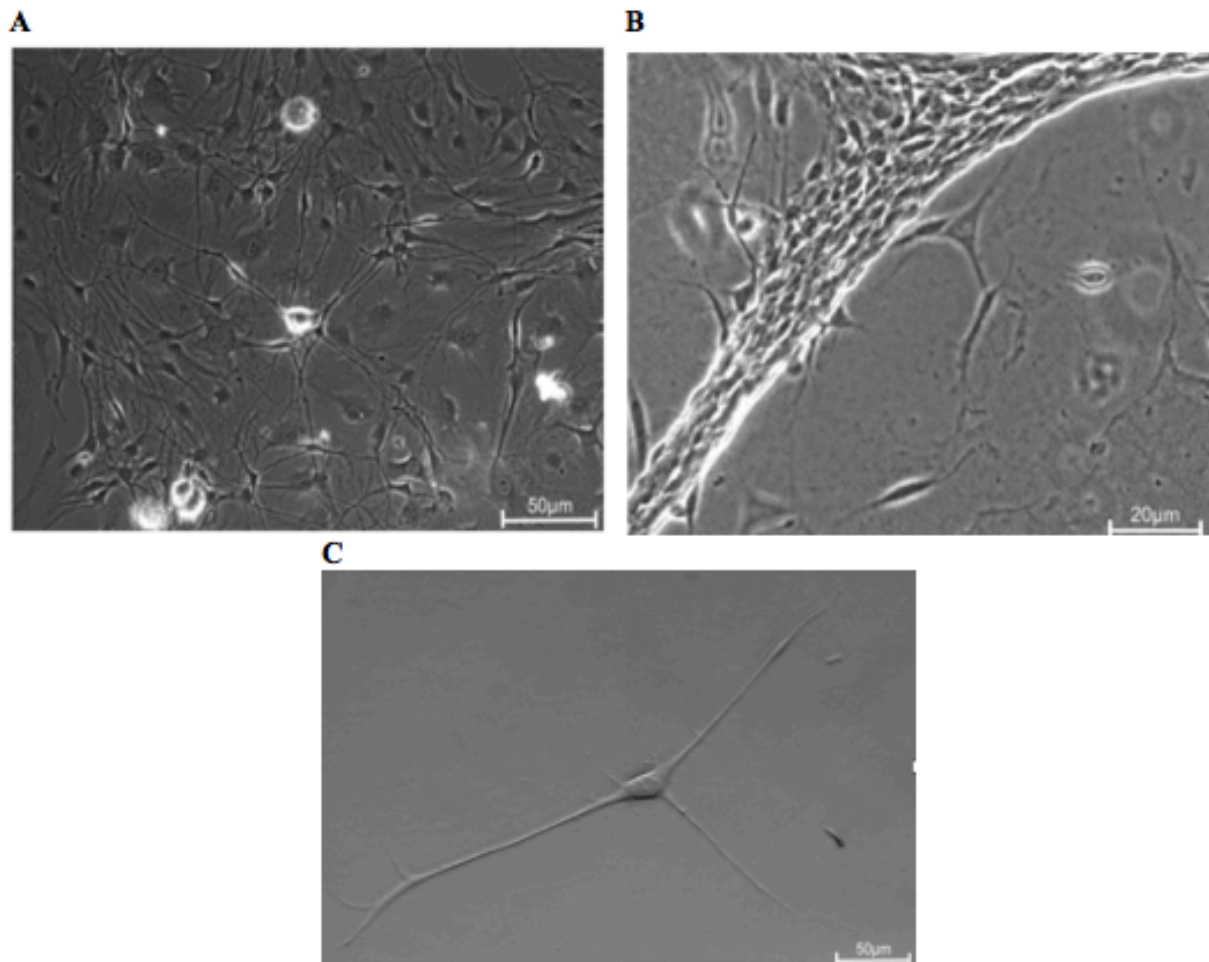
Figure 3:15 Morphological attributes of neurons grown over three types of substrate. PDL (A), Collagen (B), GelMA (C). On PDL single axons adhered to the culture dish too strongly and were thus unable to be wrapped around by surrounding SCs, (B) On collagen, a fascicle of axons emerged from the somata that were in close association with SCs but no signs of enveloping of axons were observed on this 3-D substrate. (C) Exhibits the individual neurite outgrowth over GelMA.
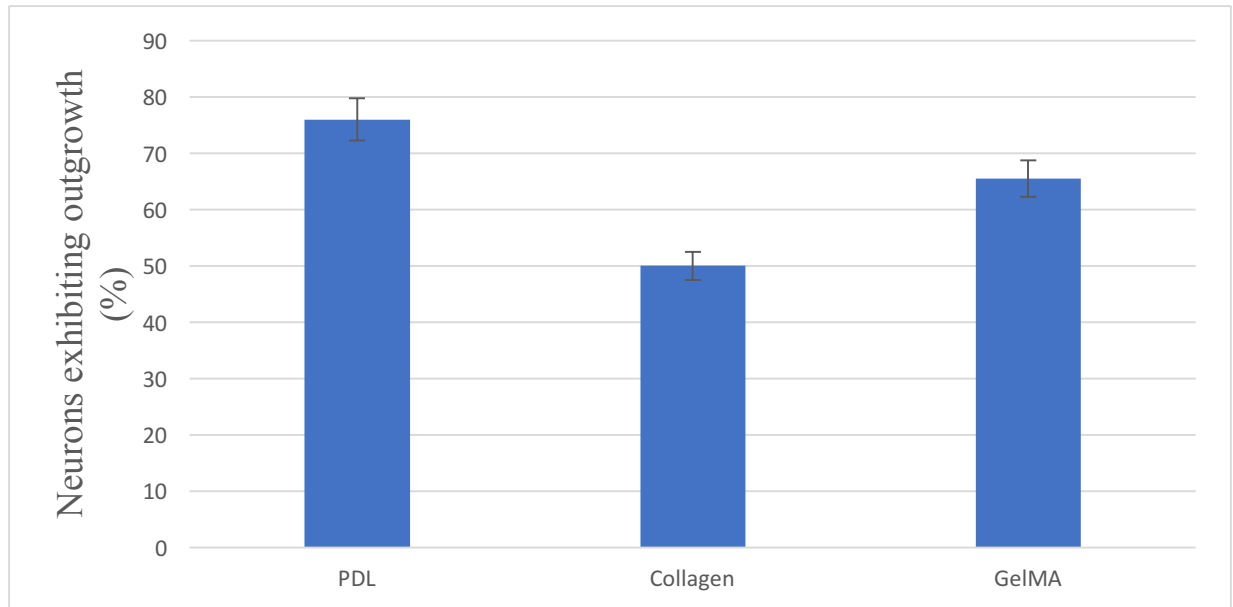
Figure 3:16 The percentage of neurons exhibiting outgrowth on three types of substrates. 75% of neurons cultivated on PDL exhibited outgrowth 42 hours after culturing, 50% over collagen and 65% on GelMA, which demonstrates GelMA serves a 3-D substrate with more biocompatible properties than collagen. One-Way ANOVA P<0.00001.
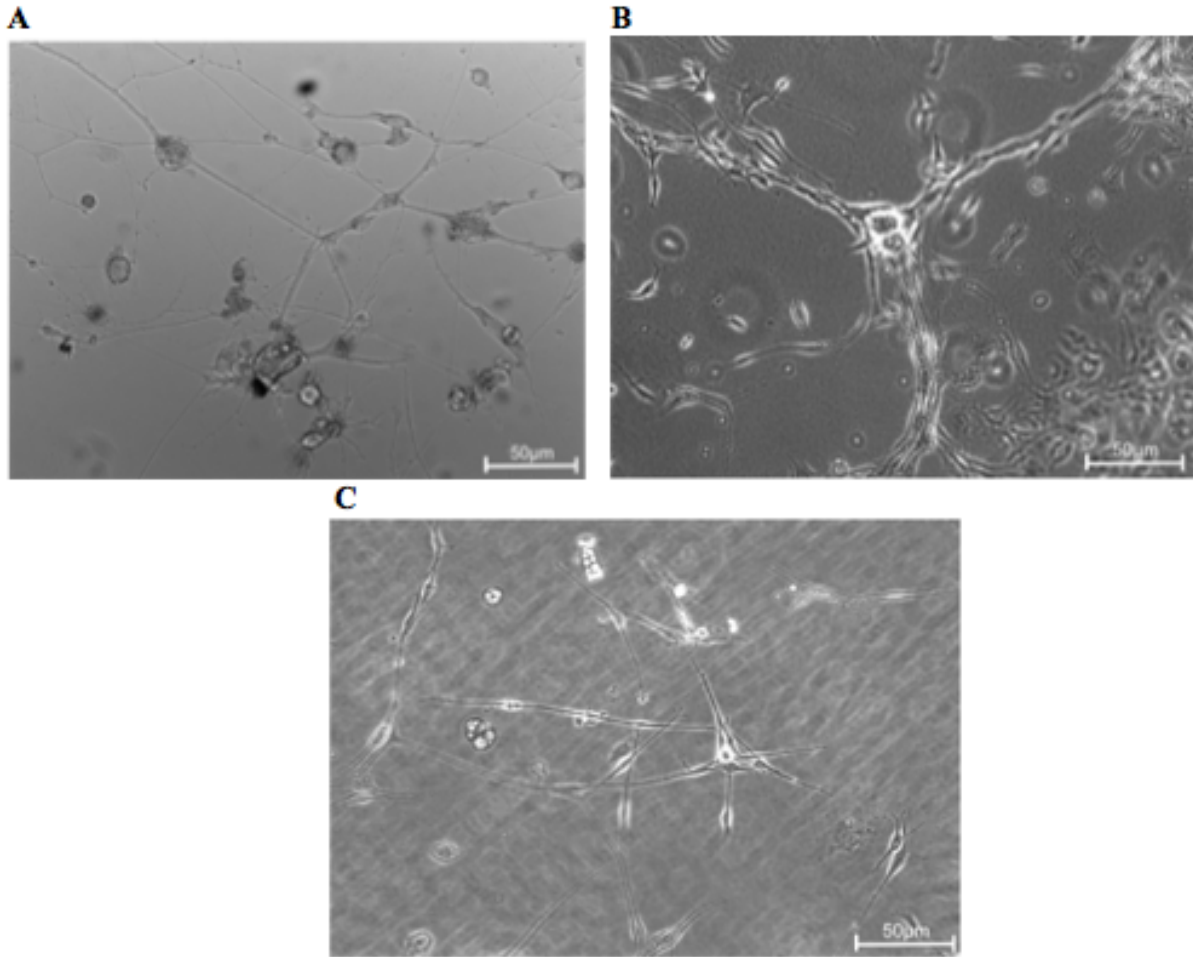
Figure 3:17 The extent of neurite outgrowth over three types of substrates. Neurite outgrowth on PDL (A), on collagen (B) and on GelMA. The photomicrographs also illustrate the 3-D configuration of collagen and GelMA, where focusing on the somata, the rest of dish becames out of focused which meant that the neurites were growing at different focal planes.
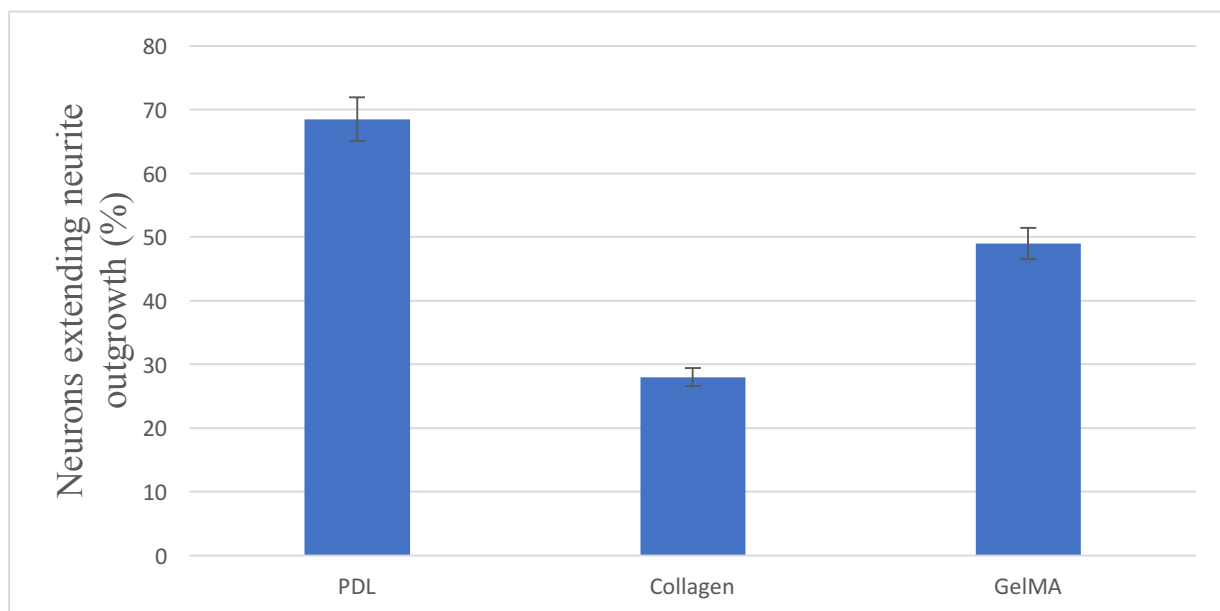
Figure 3:18 Comparision of the percentage of neurite outgrowth on three types of substrates. 68% of neurons seeded on PDL exhibited extended outgrowth, while this percentage dropped to less than 30% over collagen. Neurons cultivated on GelMA extended their neurite outgrowth up to around 50%.
One-Way ANOVA $P<0.00001$
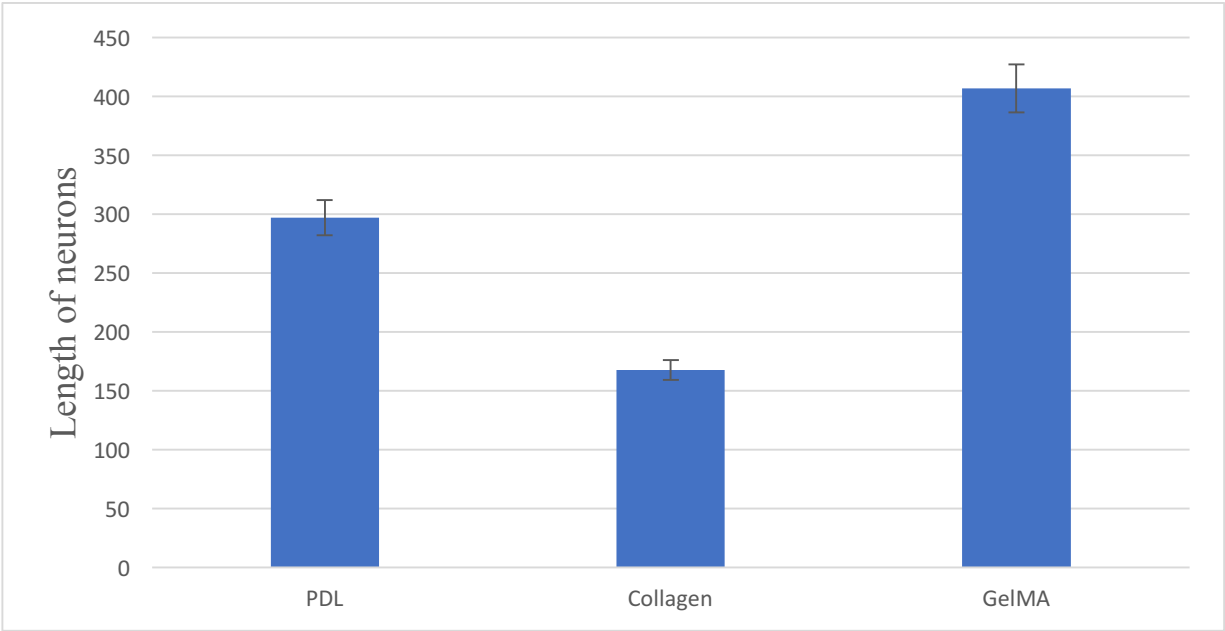
Figure 3:19 The length of neurites cultivated on 2-D and 3-D substrates. Neuronal axons on PDL as 2D substrate extended processes that were 300 μm in length, while on collagen, it was 180 μm. GelMA, as a synthetic hydrogel exhibited the longest neurite length (400 mm2) which meant that this environment provided the most suitable substrate for neurite extension
One-Way ANOVA P<0.00001.
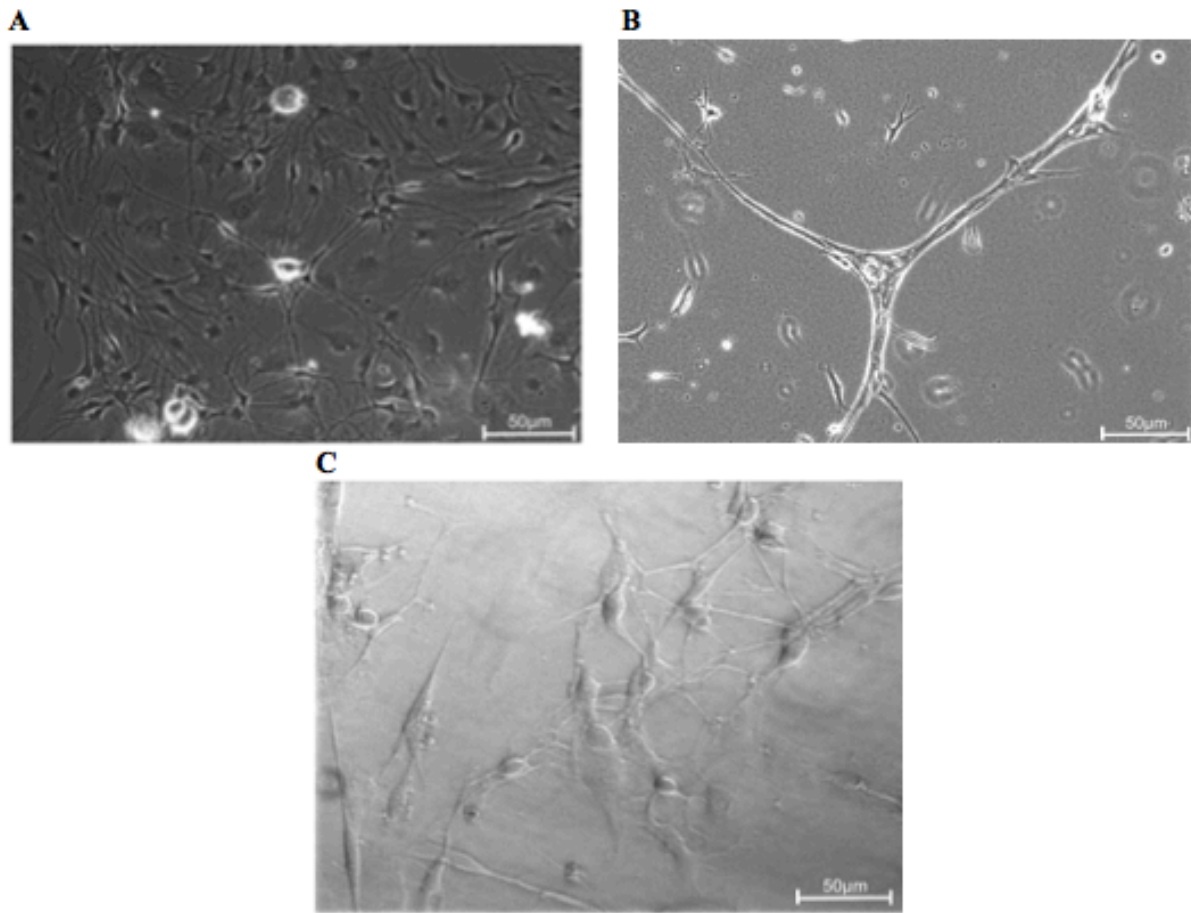
Figure 3:20 Neuronal connectivity was most pronounced on GelMA than PDL and collagen. The photographs were taken on 14 days *in vitro* over A) PDL, B) collagen, C) GelMA. Neuronal connectivity on three substrates indicated that GelMA (C) fostered more interconnected neuronal clusters at higher density as compare to both A) PDL and B) collagen.

4   Chapter 4

Aim #2, Investigating the SCs behavior and their interactions with neurons over three types of substrates.

4.1   Necessity of a 3-D scaffold for assessing neuron-SC interaction *in vitro*

Designing a culture system that could mimic myelination of peripheral nervous system (PNS) *in vitro* will not only help our understanding of Schwan cells (SC) function but also the steps involved in the process of myelination and demyelination. Here, I introduce a 3D synthetic hydrogel which acts as a scaffold for neonatal rat Dorsal Root Ganglion (DRG) neurons co-cultured with indigenous SCs. Dissociated neonatal DRGs were platted on three types of substrates, PDL, Collagen and GelMA to see how the SCs behavior changes when cultured in these extracellular milieus. PDL was considered as the control and served as a 2D substrate. Under this experimental condition, I did not see the SCs move underneath the axon because of its strong adherence to the bottom of a cultured dish. Moreover, I noted that SCs did not interact freely with the axons (Figure 1B). A 3D substrate with specific porosity was thus needed. Before proceeding in search of a suitable 3D substrate, I first sought to determine whether SC could be labeled with a fluorescent tag so as to visualize their interactions with the axons.

4.2   GFP Tagging of SCs Via Electroporation

In order to directly visualize SCs behavior over three different substrates, a cell line of SCs was tagged with GFP through electroporation technique and the changes in their morphology were investigated. I applied an electrical field to enhance the permeability of cell membrane toward green fluorescent protein (GFP). Three different dilutions (200 µL, 300µL and 400µL) were used

to receive the optimum visualization of the tagged cells. Figure 4:1 shows 400μL (Figure 4:1A) and 200μL (Figure 4:1B) dilution of GFP tagged SCs, which grew in T25 flasks.

To determine whether transfection altered the morphological properties of SCs, they were cultured on a 2D substrate where they grew very well - exhibiting spindle shape soma and shorter processes. These data thus lend further credibility that indeed the process of transfection did not alter the morphological features of SC. Next, I sought to determine whether these SC would exhibit a similar pattern of growth on collagen.

Indeed the SC grown on a collagen substrate exhibited similar morphological features to that of PDL (Figure 4:2). Their morphology, however differed when cultured on GelMA. Here, the SCs extensively spread across the substrate and extended process of much greater length on GelMA (Figure 4:3) compare to PDL and collagen. SCs moved randomly across GelMA with maximum interactions with each other; albeit they did not extend long processes in search of other cells. Here, the SCs exhibited a flat and spindle shaped morphology. Next, I sought to determine the behavior of SC when co-cultured with neurons and investigated their association with extended axons over both 2-D and 3-D substrates.

4.3   Cellular Behavior on Biocompatible Hydrogel,

Morphological parameters of SCs on 2-D versus 3-D substrate were clearly different and distinct. As mentioned earlier, DRG neurons needed to extend their neurites through different layers on a 3-D substrate to facilitate SC wrapping of the axons in a manner analogous to that seen *in vivo*.

Figure 4:4 shows that SCs survival required cell-cell interactions, that could either be from other surrounding SCs or neurons. Collagen clearly created a 3D substrate for neuronal growth and SCs had the ability to move underneath the neurite. However, due to large pore size of the

50

collagen matrix (see SEM photograph of collagen surface Figure 3:8C) I found that the cell bodies were often trapped in these groves creating clusters of cells with no specific alignment across the length of the axon. Statistical analysis on various substrates was performed by using one way ANOVA which demonstrated that the average surface roughness and process length measurement for SCs extending process over GelMA was significant $P > 0.05$.

The oversize porosity of collagen caused neurons to be trapped in between and also SCs with small size sank underneath the layers and lost their connection with axons. Under these conditions, the percentage of surviving SCs dropped and less SCs were detected as compared with GelMA Figure 4:5. PDL exhibits maximum survival percentage, around 90%, this amount dropped to 55% on collagen and then increased to around 70% on GelMA which indicated that GelMA provides a better substrate for SCs survival compare to that of collagen.

4.4   Schwan cells process extension on Gelatin Base Hydrogel (GelMA),

Figure 4:6 shows the behaviors of SCs on three types of substrates, (PDL, as 2-D and Collagen and GelMA as 3-D environments). Clearly the SCs seeded on both Collagen and GelMA extended their processes but there were significant differences in the morphological activity between two types of 3D substrates. SCs over collagen exhibited similar morphology than that of a 2D environment but with much less density. SCs on PDL and collagen revealed soma spindle shape morphology with shorter process, while SCs over GelMA exhibited different structure. Specifically, GelMA with higher contact surface area provided better substrate for SCs attachment and their process extension. Specifically, SCs were in contact with each other either at the end of their process or middle of their length (Figure 4:7).

However, the SC exhibited longer cellular process extension on GelMA which suggested that the higher contact surface with suitable porosity may have helped the SCs survival and better health under these experimental conditions to that of either collagen.

4.5   Schwan cell - Neuron Interaction

To visualize neuron – Schwan cells interaction at the level of single axons, DRGs were co-cultured with SCs on three types of substrates including PDL, Collagen and GelMA. As mentioned earlier, PDL served as a 2-D substrate, which did not promote axonal wrapping by the SCs. By manipulating gelatin concentration and also functional group cross-linking time, I sought to further develop the GelMA substrate. After 14 days of *in vitro* culturing, Myelination process was induced by adding 50 µg Ascorbic Acid (AA) to the DRG media and to determine how the SCs – neurons interaction changed. Figure 4:8 shows SEM photographs of neurons cultured on GelMA and indicates neuron – SC interaction at the level of single axon. Figure 4:8A shows that a multitude of axonal processes emanated from the soma and these were wrapped up by SCs (Figure 4:8B is a higher magnification of neurons that enveloped by SC).

In order to demonstrate that SCs were indeed in contact with neurons and expressing myelin specific proteins, I used a specific set of antibodies. The first antibody that I applied was neurofilament (NF), which is expressed along the length of an axon**.** Figure 4:9 shows the neurofilament expression across the length of axons in collagen and GelMA environments. Due to single axon configuration on GelMA, which could be deduced under the microscope, the immunostaining with NF showed the SCs – neurons interaction was clearly discernable; such interactions were not observed on either PDL or collagen.

NaV1.6 and CASPR antibodies were also used to localize Na^+ and K^+ channels that are the hallmarks of the nodes of Ranvier. These two antibodies were applied on different dilutions, but failed to reveal a clear demarcation of these ion channel proteins. Instead of detecting Nodes of Ranvier proteins, Myelin specific antibodies such as myelin basic proteins (MBP) and myelin associate glycoproteins (MAG) were then perused.

4.6 The expression of Myelin Basic Protein

As mentioned earlier, neural crest cells are the origin of immature SCs, which then differentiate into two different types of mature SC population, Myelinating and Non-myelinating SCs (Jessen & Mirsky, 2005). Myelinating SCs sheath large-diameter axons while the nonmyelinating SCs envelop small diameter axons. Some transcription factors including Krox20, Oct6 and Sox10 are deemed important in SCs differentiation (Stolt & Wegner, 2016; Svaren & Meijer, 2008; Monk, et al., 2015).

Because mature SCs extended their process that came in contact with other SCs and also the neurite, I initially focused on three antibodies including neurofilament, MBP and MAG. Figure 4:10 shows a higher magnification of neuronal cell body, which extended its neurite, NF (red) is expressed along the axon. When the axon is enveloped by a mature SC, the MBP is also co-expressed (green). This pattern of staining demonstrates co-localization of single axon over GelMA and its association with SC.

Figure 4:11A shows the cell body stained with NF and its extended axons at lower magnification. Here a single axon grown on GelMA appears to interact directly with SCs. In Figure 4:11B, I observed the red color of NF, which turned to green when stained with an MBP antibody. Single axons grew in different directions and were in contact with "myelinating" SCs.

4.7   The expression of Myelin Associated Glycoprotein

With a phenotypic marker, I could recognize SC behavior at different developmental stages including both non-myelinating and myelinating SCs phenotype (Jessen, et al., 1990). Microenvironment signaling is important for SCs responsiveness which helps shape their polarity, specific function and behavior. Receiving signals from axon is the primary regulator for SC's functional expression of various proteins. Different parts of a neuron come in contact with SC including the cell body, extended neurite and dendrites. Depending on which part was in association with glia cells, SC function was expected to change. Adhesion of SC to the extracellular matrix (ECM) plays an essential role in morphogenesis. My observation from *in vitro* studies indicated that SCs grown on GelMA behaved differently from those cultured on collagen. Figure 4:12 shows that the extended neurites stained with NF grow in various directions and that GelMA 3D substrate provided an anchor for axonal growth. Axons interacted with SCs to simply en-sheath the myelinated and unmyelinated nerve fibers. In Figure 4:12 nuclei of the SCs marked by Dapi appeared blue and revealed the site of neuron – SC interaction. Green dye on the other hand, represented myelin associated glycoprotein (MAG) that was expressed in SCs interacting with the axons.

Figure 4:12B at a higher magnification shows how the SCs (in green color) starts to wrap around the axons, which were stained with NF and by enveloping the SCs – with overlapping colors, the red turns to green.

In summary, in this chapter the neuron – SCs interaction were assessed to determine the difference between SCs behavior on collagen and GelMA as a 3-D synthetic hydrogel. Whereas collagen is a 3-D substrate with higher porosity diameters, I could not observe the neuron – SC

interaction at the level of single axon. Due to the fact that collagen did not provide suitable contact

surface, the SCs were sunk in deeper layer and unable to interact with single axons.
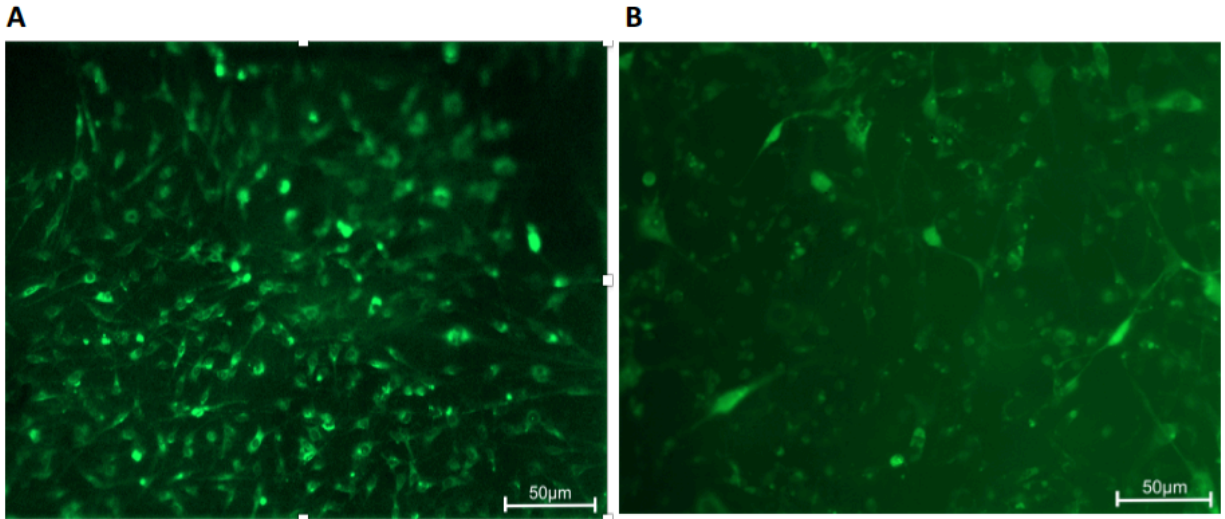
Figure 4:1 To visualize the SCs morphology on PDL, SCs were cultured on PDL with A) 400μL and B) 200μL dilution of GFP tagged SCs via electroporation. SCs are in spindle shape with shorter process in higher density (A), while at lower density (B), they extend their process towards the other cells.
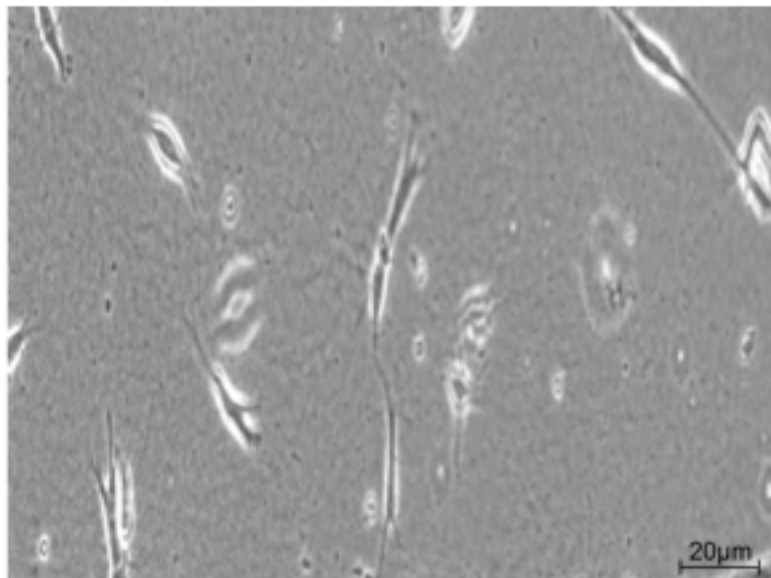
Figure 4:2 SC cultured over a period of three days on collagen substrate. SCs show similar behavior to those grown on a 2D environment; spindle shape nuclei with short process are discernable.
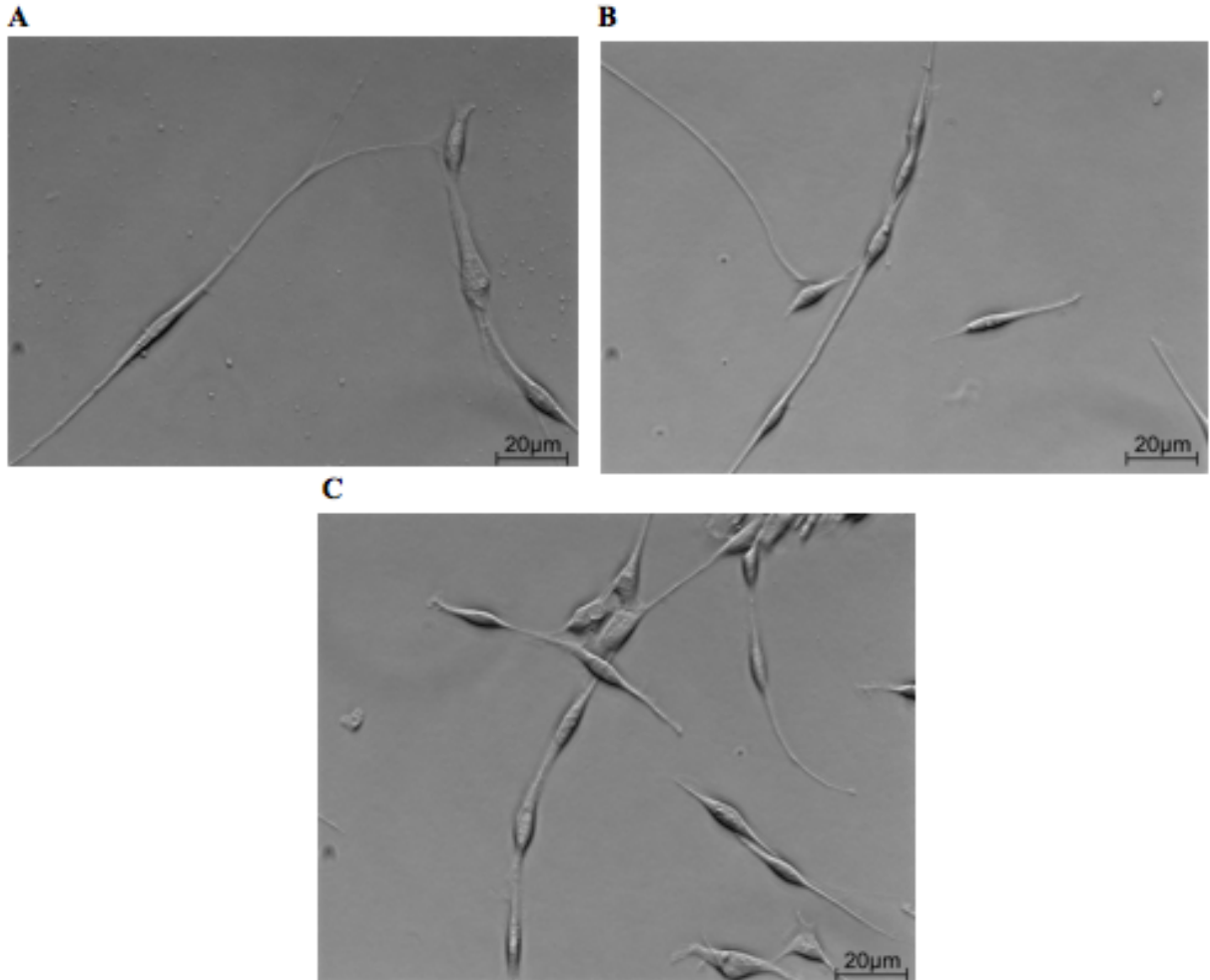
Figure 4:3 SCs grown on GelMA extend their process and came in contact with other SCs. A) indicates a SC that extended its process to reach out a line of SCs along each other from end to end. B) Shows that a closer SCs extended shorter process as compared to SCs that had a longer process. C) This photomicrograph shows a 3D culture environment where SC move in different layers and adhere to each other in clusters/ rows.
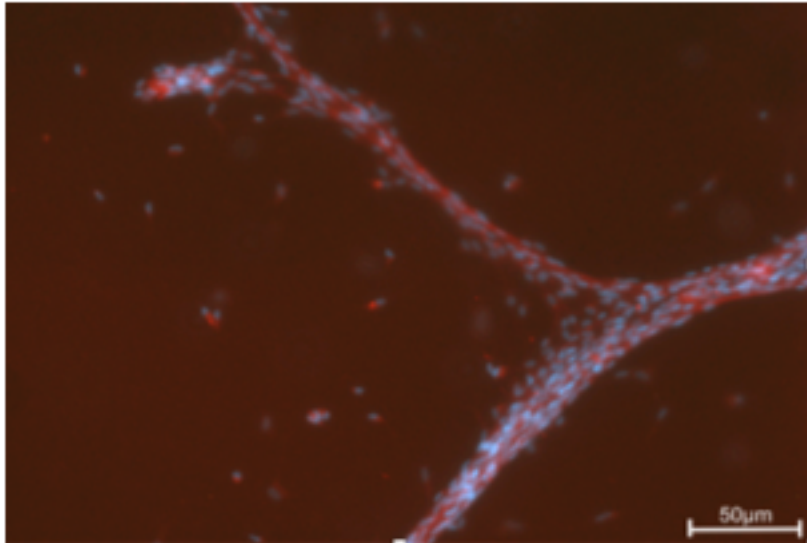
Figure 4:4 SCs survival requires cell-cell signaling that can either be from other surrounding SCs or axonal neurites. Hundreds of SCs (DAPI, in blue color) that were attached to the neurites (red) exhibited healthy behavior while individual SCs that were not in associate with neurite, did not survive.
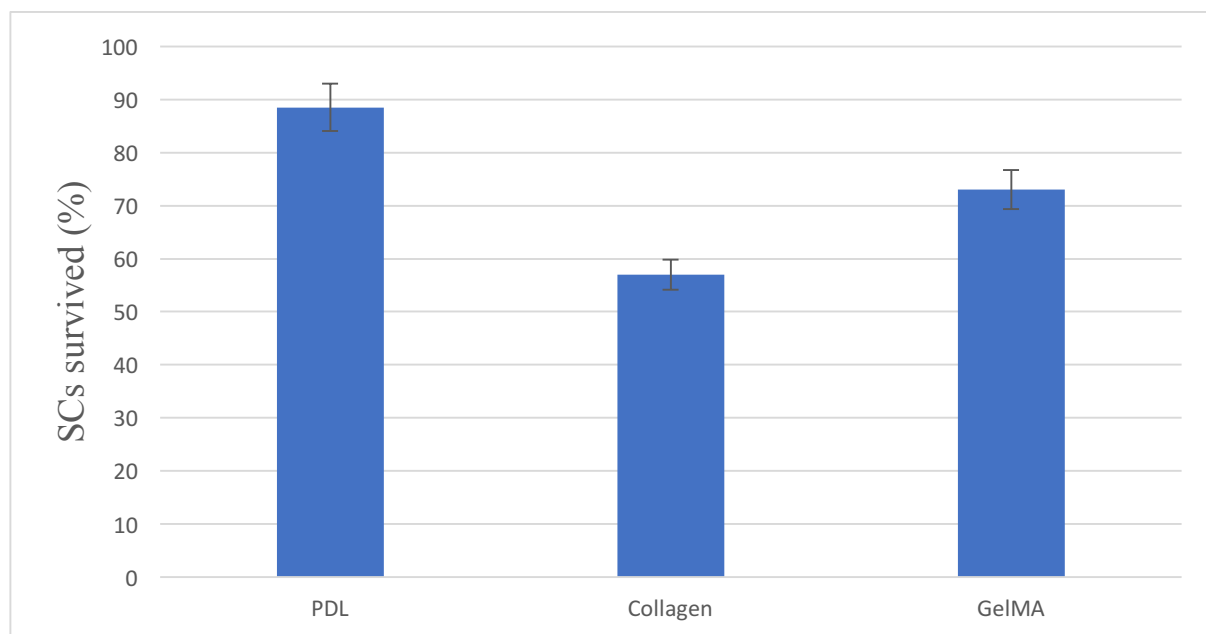
Figure 4:5. Shows the percentage of SCs survival on three types of substrates. 350 SCs seeded on PDL exhibited 90% (320) survival behaviour, while their numbers dropped to 55% (200) on collagen. 73% (260) of SCs survived on GelMA which endorses the prosity of this 3-D substrate as most approperiate for SCs survival. One-Way ANOVA P<0.00001
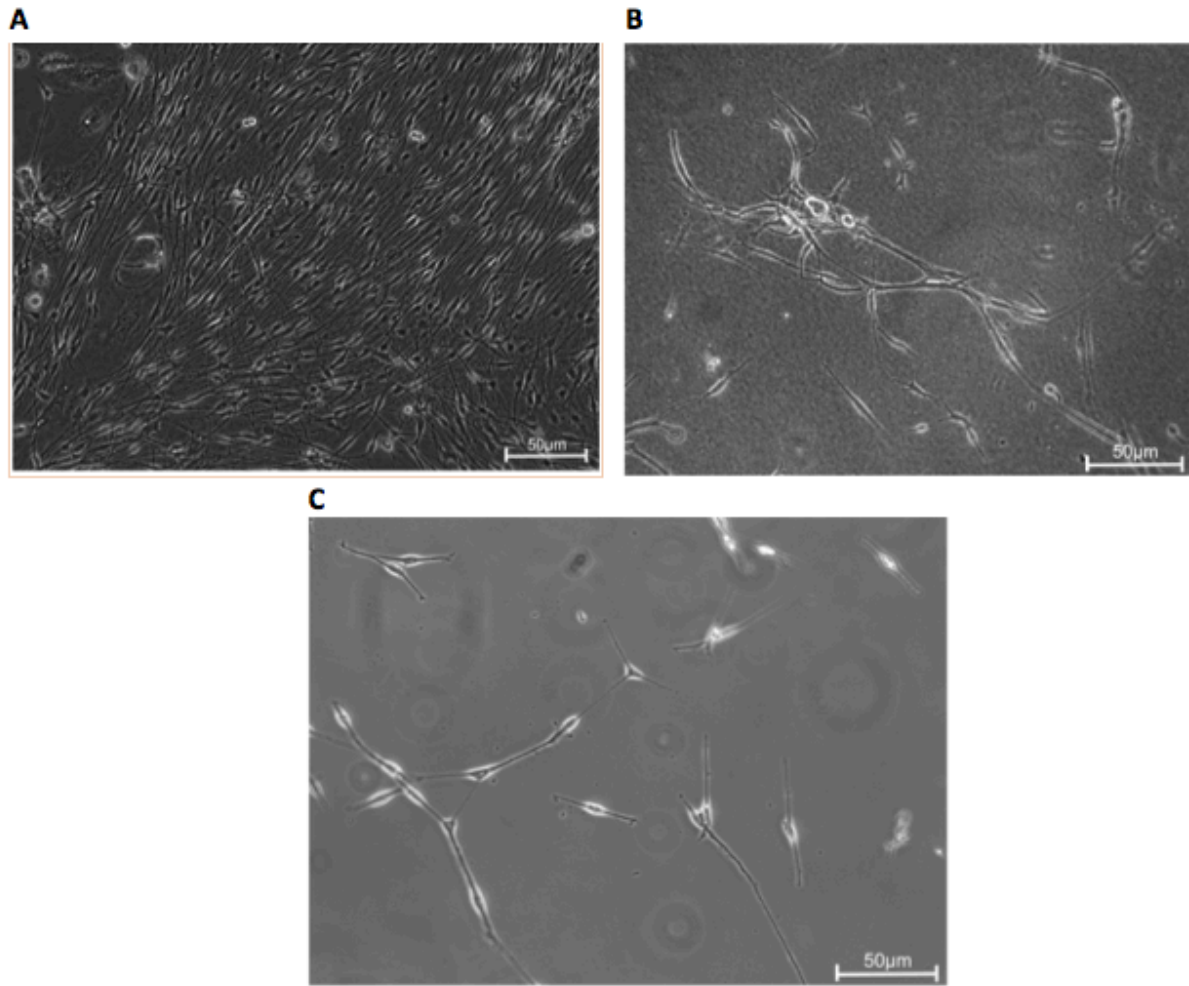
Figure 4:6 SCs behavior over three types of substrates A) PDL, B) Collagen, C) GelMA. SCs on PDL show higher survivability with normal morphology such as spindle shape cell body with shorter process. As a similar behavior was observed on collagen (B) (flat cell body with short process). On GelMA (C) the process became longer with different morphological features compared to collagen and PDL. SCs on GelMA extended their process to reach their neighboring SCs.
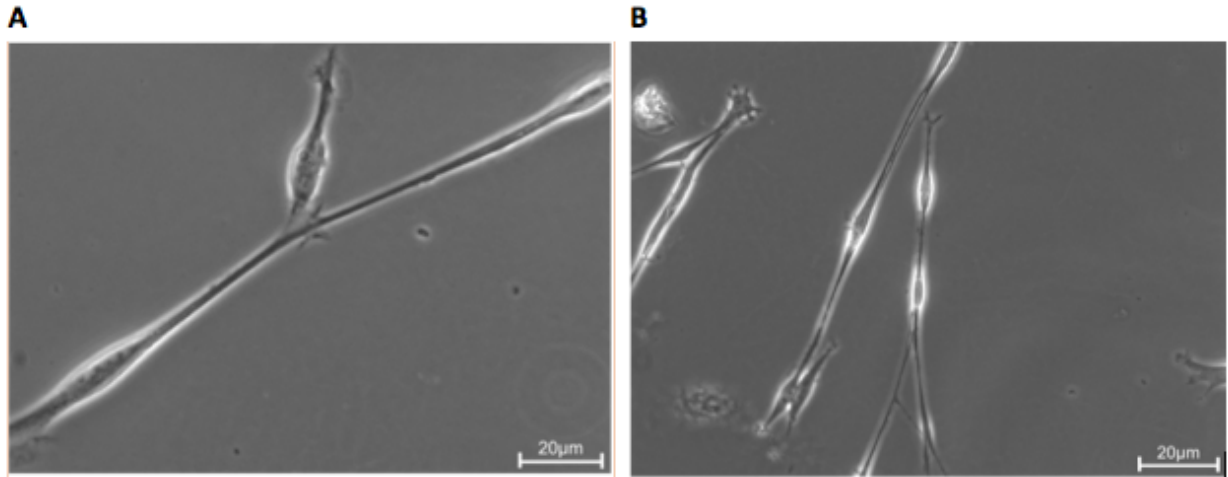
Figure 4:7 SCs cultured on GelMA make contact with individual and groups of SCs that come in contact and adhere along their process.
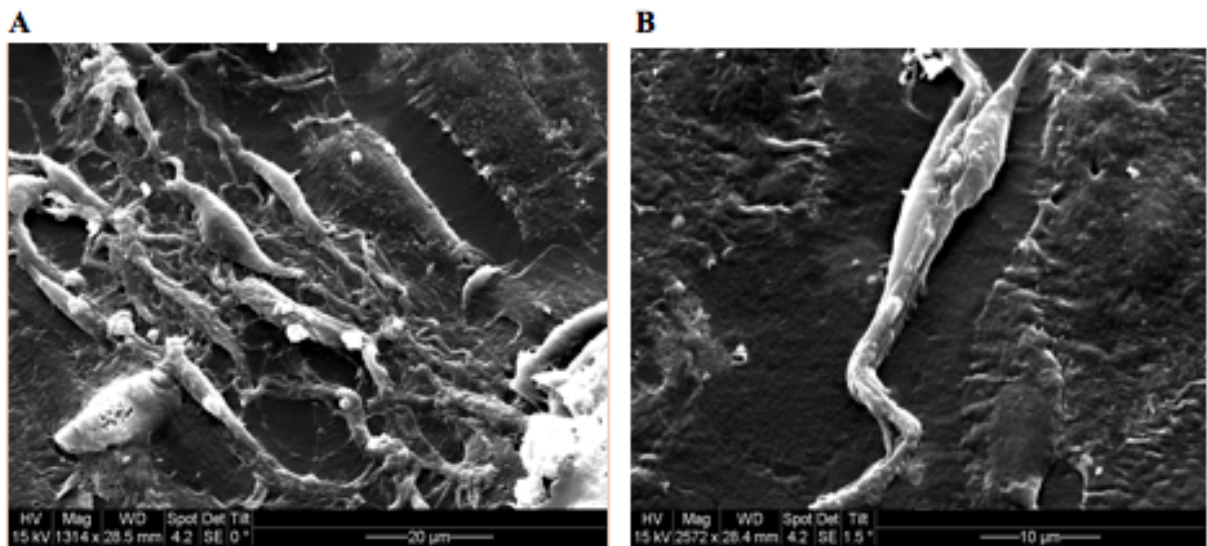
Figure 4:8. A) SEM photographs show neuron – SCs interaction. A) Indicates the multiple single axons emerged from somata were in contact with SCs, B) indicates SCs wrapped up a neurite at a higher magnification.
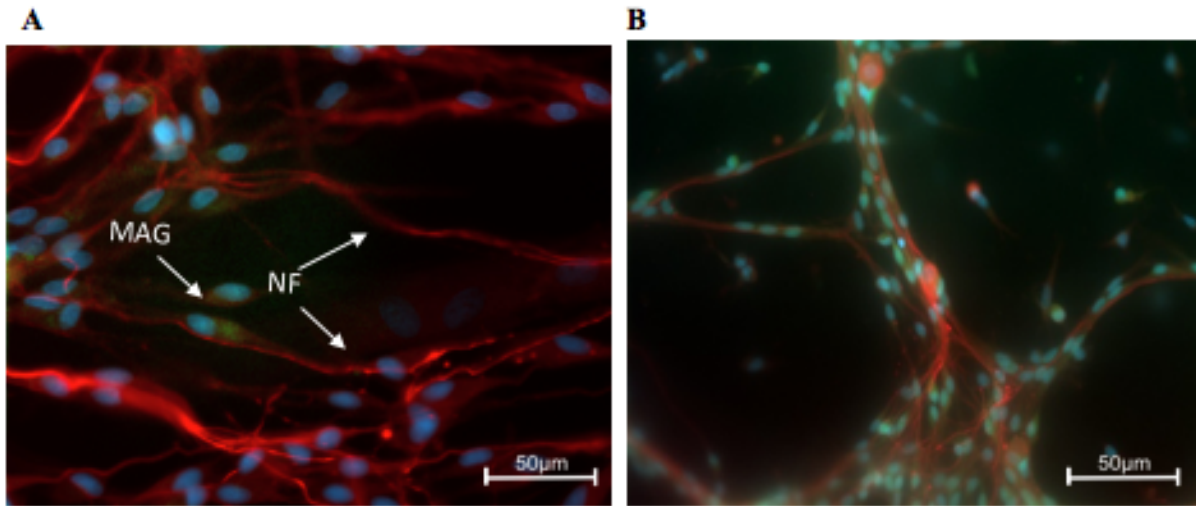
Figure 4:9. A) Neurites grown on GelMA were stained with NF indicating the extension of single axons which were in association with individual SCs, B) fascicles of axons extension over collagen indicated hundreds of SCs were in contact with axons with no obvious interactions with axons.
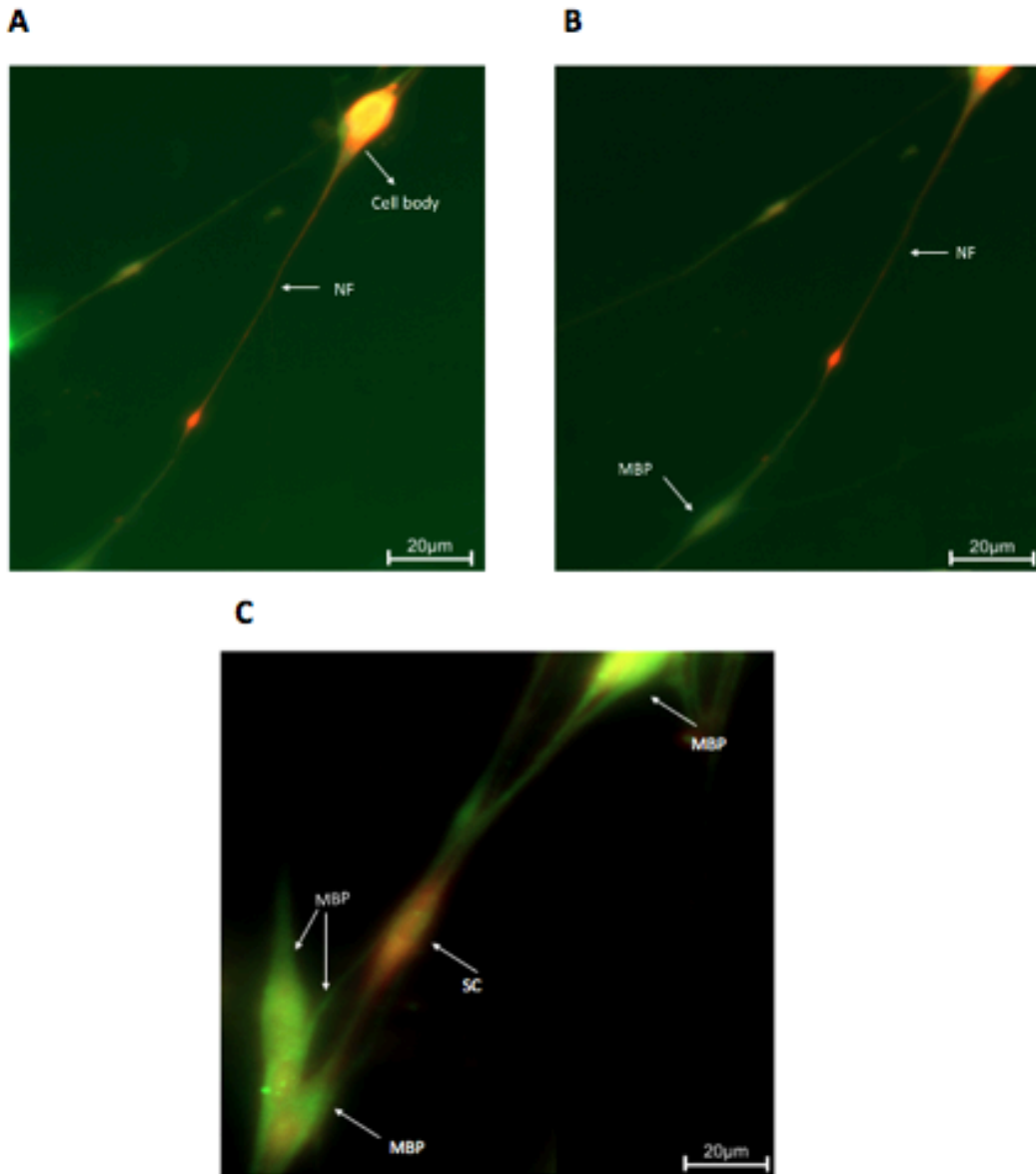
Figure 4:10. The interaction between neurite extended and SCs are shown here. A) Shows a higher magnification of neuronal cell body, which extended its neurite, NF (red) is expressed along the axon. When the axon was enveloped by a mature SC, the MBP was also co-expressed (green). This pattern of staining demonstrates co-localization of single axon over GelMA and its association with SC.
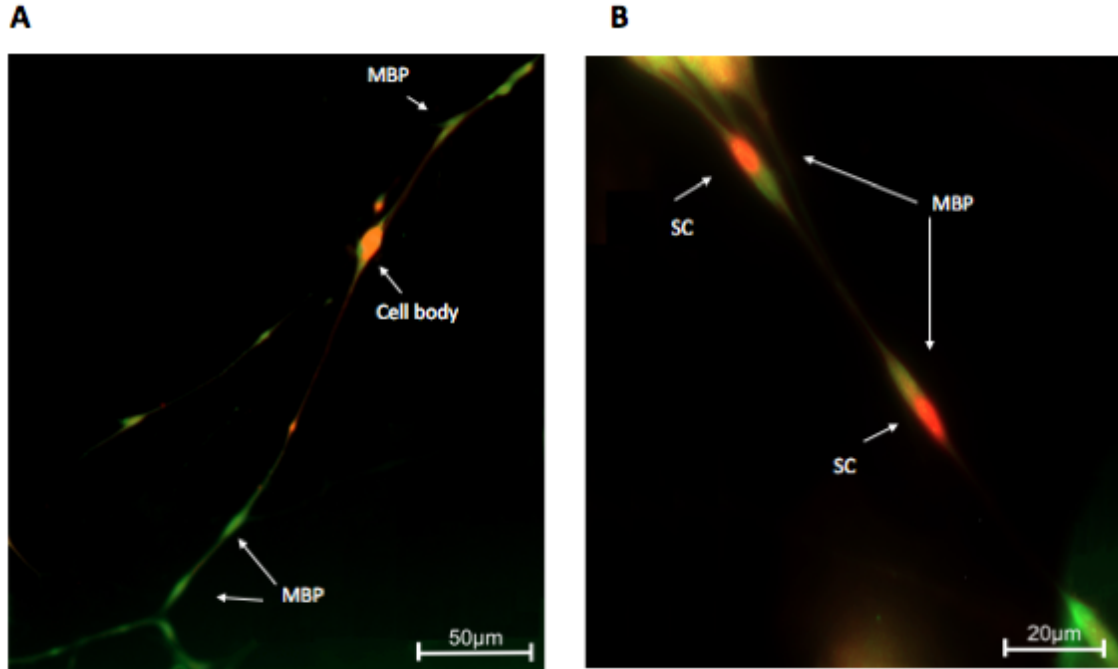
Figure 4:11 Illustrates the expression of MBP and NF along the axon. A) indicates the cell body stained with NF and its extended axons at lower magnification and (B) higher magnification. Here a single axon grown on GelMA appeared to interact directly with SCs.
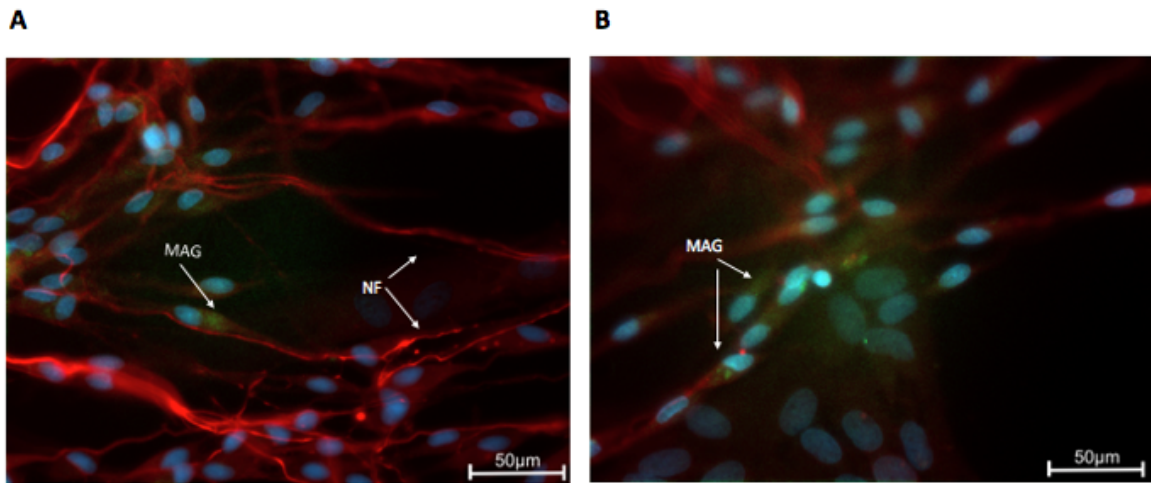
Figure 4:12 . Indicates the single axons emerged from somata on GelMA which was stained with NF(A) shows how the SCs (in green color represent MAG) starts to wrap around the axons by expressing MAG in green color. The nuclei of the free SCs around axons marked by DAPI appeared blue which means they are in immature SCs stage and they are not expressing MAG. B) Green dye on the other hand represented myelin associated glycoprotein (MAG) that was expressed in SCs interacting with the axons.

5   Chapter five: Discussion

Defining the cellular and molecular mechanisms underlying brain development, plasticity and regeneration are often difficult to decipher in the intact brain which harbors billions of neuron, trillions of glia and even a large number of synapses. To tease apart various steps by which neurons acquire their mature polarity, cell-cell interactions and synaptic wiring, a variety of *in vitro* and *in situ* models have been developed for both vertebrate and invertebrate neurons (Getz, et al., 2018).

In the present study, I took the *in vitro* approach a step forward whereby a novel 3D substrate was developed and exploited to gain insights into neuronal growth and how they interacted with their corresponding and supporting SC. I demonstrated that whereas both 2D (PDL) and 3D substrates such a collagen and GelMA supported growth, survival and neuronal outgrowth from DRG neurons; the later however acted more in a manner analogous to that of *in vivo*. GelMA thus offers a wonderful opportunity to serve as one of the most suitable substrate for defining the steps underlying myelination and demyelination in the peripheral nervous system. The lessons learned from this model could thus be applied to seek commonalities and differences as they pertain to myelination in the CNS.

To develop a model system which mimics *in vivo* situation for *in vitro* studies, DRGs were cultured on 2-D (Ply- D-Lysine PDL) substrate that supported neuronal survival and fostered neurite outgrowth. DRG neurons seeded on laminin coated dishes (PDL) exhibited high rate of survivability and process extension. The interaction between the DRG neuronal process and the indigenous SCs that were often isolated with DRGs during dissection procedure were however futile as the model did not permit interactions between these two cell types. Specifically, even though I did see that the SC came in contact with axons but because of their attachment with the substrate, a wrapping around was improbable. I anticipated that under these conditions if a SC

were to wrap an axon that it would in its detachment from the surface and hence the collapse of the neuronal architecture. Whereas, both the survival rate and the extent of growth was more robust on PDL, this substrate which is one of the most exploited for *in vitro* studies, would not have served the purpose of my studies. Based on its neuronal survivability and growth promoting capabilities, PDL was thus used as one of my controls and as a benchmark for other substrates.

I next moved on to use a 3D substrate where axons would not be firmly stuck to the substrate and suspended in the matrix thus providing an opportunity for axons to wrap around them. Collagen was chosen for this purpose. Although neurons both survived and extended processes on collagen, they tend to cluster and bundle around the axonal process. I also noted that multiple axonal process emerged from cell body and also fasciculated into larger clumps thus making it impossible to visualize individual axon – SC interaction. Axonal clumping and bundling is often observed *in vitro* when neuronal processes tend to prefer their adjacent counterparts for adhesion and growth as compared with the substrate on which they are grown.

A similar process is seen *in vivo* when all subsequent axons tend to stick to the "pioneer" axons through cell adhesion molecules thus giving rise to nerve bundles. Thus, it would appear that the axons emanating from the DRG neurons tended/preferred to stick to their neighbouring axons as compared to the collagen substrate. Another reason may be that when I examined the collagen substrate under the Scanning Electron Microscope (SEM), I found the structure to be too porous with large interstitial gaps, which could have served as a deterrent for axonal growth thus resulting in clumping. It would thus appear that the initial axons may have served as a scaffold facilitating the growth of all subsequent axons. This observation raises an interesting possibility that in the future nano-tube scaffolded into collagen or GelMA could provide a wonderful opportunity to study cellular migration and growth. The porous nature of collagen although did

not suite for my studies but this feature could perhaps be an advantage when designing a novel matrix, which could be a hybrid of nano-tubes and this substrate. This potential utility would however need to be explored further in the feature. Whereas collagen supported neuronal survival and growth, the clumping together of axons made it rather difficult to decipher direct interactions between the SC and the DRG axons.

In search of yet another 3D substrate, I explored GelMA as a potential substrate. By manipulating the degree of polymer and photo-initiator concentrations, photo-crosslinking time and UV light intensity, the optimized condition for neuronal growth was eventually obtained. The survivability, biocompatibility, the ability of three substrates (PDL, collage and GelMA) to produce longer neurite outgrowth were than compared to achieve the optimum substrate for neuronal outgrowth. I also examined the SCs behaviour on both 3-D substrates and observed the interesting behaviour of SCs on GelMA as compared to collagen. I found that the SCs processes were longer and they also exhibited the higher rate of survivability and looked healthier on GelMa than collagen. To validate GelMA's potential as a preferred substrate, it was deemed imperative that I compare and contrast its qualities with collagen in details.

5.1   PDL - collagen Comparison

After culturing the DRG neurons on Poly-D-Lysine for several days, although a number of cells floated away, a larger contingent however remained attached on PDL (Renault, et al., 1995). There are several reports that suggest that the efficacy of a cell's metabolism may affect their morphology and cytoskeletal structure both *in vivo* and *in vitro* studies (Alberts, et al., 1983). Similarly, other studies using Poly-D-Lysine as a potential substrate have shown that this rather "unnatural" substrate does indeed enhance several aspects of cellular differentiation and growth (Watson, et

al., 1987). These data are nevertheless consistent with other studies where DRG neurons when cultured on PDL exhibited high rate of survivability (Bouffi, et al., 2010) due to the positively charged PDL surface that leads to firm attachment of cells on this 2-D substrate. The reasons for a larger proportion of cells failing to adhere to the substrate could have been due to enzymatic treatment which may have overly digested the cellular membranes hosting the receptors required for cell attachment. Interestingly, the measure of the neurite extension associated with cell adhesion properties of PDL substrate, could be further used to improve the biomimetic conditions for *in vitro* studies (Bouffi, et al., 2010).

Neuronal culture environment and media supplement including F11 demem media, B27, FBS and NGF are important factors to recapitulate *in vivo* situation for neurite and synaptic formation, neurotransmitter release (Bottenstein & Sato, 1979; Romijn, 1988), and in this study myelination. This study thus suggests that in addition to a suitable substrate an appropriate growth media is also essential to enhance the survival and growth rate of neurons. In previous studies, Neurobasal media was the main component for cell culture studies (Chen, et al., 2008), but I observed better results vis-à-vis neuronal survivability and longer axonal process in F11 Demem media compared to neurobasal media. What I observed on PDL as 2-D substrate was predictable due to the attachment of neurons at the bottom of cultured dishes, but native SCs that accompanied DRG neurons during cell isolation could not move underneath the neurites and were unable to wrap the axons. As I mentioned earlier, this may have resulted from the nature of the substrate that holds a positive charge produced by PDL and thus charge shielding may have been disrupted as the SC infiltrated the contact points. Taken together, my data show that whereas PDL is indeed a viable model to study neuronal viability and growth, it may not serve the purpose when it comes to studying neurons to SC interactions.

5.2   Cell viability and survival on PDL and collagen

Collagen is a naturally occurring, three-dimensional substrate which contains an extracellular matrix microscale fibers with micro and nanopores suitable for neuronal attachment (Gelain, et al., 2006) and neurite outgrowth. To ensure that collagen indeed creates a porous scaffold which is growth supportive and does not compromise neuronal viability, I used commercially available collagen. The SEM photograph of collagen (Figure 3:8 C) revealed that a 3D matrix was successfully created with appropriate porosity for neuronal growth. The porosities within collagen facilitated neuronal growth traversing through different focal planes and allowed SCs to be freely suspended around neurites. This 3-D environment not only provided substrate for cell attachment, but also allowed diffusion of oxygen, hormones, secreted neurotransmitters and nutrients, as well as removal of debris and waste products (Gelain, et al., 2006).

As we know that in the intact organ tissue, the movement of cells in a 3-D milieu follows signaling from their neighbors and surrounding cells including chemical signals and molecular gradients and also micro-environmental signaling from the extra cellular matrix itself which is essential for organism development (Lanza, et al., 2000). Similarly, in the present study, I found that collagen not only supported survival but also enabled axonal growth to occur which traversed through several layers of the scaffold. Compared to PDL, I found a lower rate of neuronal survivability (60% on collagen as compared with 80% on PLD). I consider this lower survival rate to owe its existence to the fact that collagen may have acted as a barrier for various diffusible factors present it the culture media. This postulate could not however be tested experimentally in this thesis and could be the focus of future studies. Another factor contributing to low survival rate may be the mechanical properties and contact surface areas of PDL verses collagen. The stiffness and rigidity of collagen decreased and it produced a flexible substrate and this might have affected

neuronal attachment. As we know, cells prefer the rigid surface to adhere where they attach firmly rather than an unstable substrate; this may explain as to why fewer cells survived on this substrate.

Furthermore, collagen has the microfiber structure with a size of 10-100 μm which is similar or bigger than most cells (5-10 μm) (Palsson, et al., 2003; Tuzlakoglu, et al., 2004). As compared with collagen substrate the cell body,(Figure 3:14) exhibited a more bulky somata appearance with tiny sprouts which could be due to the fact that the neurons were either trapped in the pores or sank into the deeper layer. The somata on the PDL however, looked more flattened shape with neurites that extended in all directions.

The fact that neurons survived better on PDL rather than collagen could be because of perturbation of N-Cadherin and neuronal cell adhesion molecules (NCAM) functions on collagen that control the neurite generation (Bixby, et al., 1987; Seilheimer & Schachner, 1988). Cadherin's are known as cell-cell adhesion receptors which bind cells and play an important role in maintaining intercellular connections (Takeichi, 1991). Whereas N-Cadherins are involved in Ca^{+2} dependent cell–cell adhesion mechanism and exist in intact tissues, thus a tissue engineered substrate is needed to provide situation for these cell adhesion molecules activity. Any deficits in cadherin activities may cause dissociation of cell layers and eventually inhibition of neuronal outgrowth and cell death (Takeichi, 1991). Although collagen created a 3-D substrate for neuronal growth, it might not have sufficed to provide a suitable cadherin's activity as compare to PDL thus leading to decline in the cell attachment, cell-cell interaction and leading to cellular morbidity.

5.3 Fasciculation of axons on collagen

One of the main aims of this study was to develop a 3-D substrate for neuronal growth to study the DRG myelination at the level of single axon, but it would appear that neurites on a collagen

substrate preferred to fuse with each other – rather than growing in different directions. This fasciculation may have thus made it difficult for SC to wrap around the axons.

In vivo studies suggest that the pattern of axonal fasciculation is controlled by two classes of proteins: classic cell and substrate adhesion molecules (CAMs and SAMs, respectively) (Van Vactor, 1998). It has also been reported that the axonal fasciculation is directed by "contact action" of selective adhesiveness on various axon surfaces (Van Vactor, 1998). Mutation in both proteins in *in vivo* studies indicated the deficits in fasciculation (Van Vactor, 1998), which may suggest that this type of proteins is likely important for axonal fasciculation.

Figure 3A shows the bundle of axons that grew on collagen and hundreds of SCs that were attached to the neurites. When the single axon is not discernable, the SC-Neuron interaction is not readily observable. What I noted in this environment was that when the SCs were in contact with neurites, they survived longer, suggesting that the SC survivability is enhanced in instances where they maintain contact with their corresponding axons – irrespective of the fact that they were not involved in myelination.

In order to avoid programmed death cell (PCD) (Raff, 1992), the developing vertebrate cells are required to receive signals from their neigbours. It seems that the SCs contact with axons, axonal neurites may have received the survival signal from the axons though the nature of this survivability factor remains to be determined. As mentioned in chapter 1, neuron-derived neuregulins (NRGs) signals promote survival and differentiation of SCs precursors (SCP) in order to convert them to mature SCs and myelin segments (Cheng, et al., 1998). In addition to NRGs, basic fibroblast growth factor (bFGF) and insulin-like growth factors (IGFs) also transiently enhance SCs precursors survival rate (Jessen, et al., 1994 ; Syroid, et al., 1996).

The idea of SCs survival during the early stages of development was supported by *in vivo* studies (Jessen, et al., 1994 ; Syroid, et al., 1996). Here, *in vitro* data serve to suggest that regardless of their wrapping, physical contact between axons and the SC enhances their survival rate. These data thus provide the evidence that indeed neurons-SC contact can influence and enhance the survival rate of SC – irrespective of their functional association with axon.

The main objective of this study was to determine axonal myelination at the level of single axon *in vitro,* thus a 3-D substrate that provides fascicle of axons into tight bundles would not be appropriate, for solving this problem and for obtaining the individual neurite process. I thus developed a 3-D gelatin base hydrogel with tunable chemical and mechanical properties that could produce a biocompatible substrate. Dependence of the hydrogel compositions on polymerization methods and their crosslinking density caused gelatin base hydrogel become a versatile platform to provide desired combinations for the required applications (Annabi, et al., 2014).

5.4   Biomedical application of GelMA scaffold

A 3-D scaffold must resemble the characteristic of native extracellular matrix (ECM) to develop support for cellular growth and tissue formation (Alge & Anseth, 2013). Observation of cell–cell and cell matrix interactions is thus necessary for designing a biocompatible 3-D synthetic hydrogel scaffold. Gelatin Methacryloyl (GelMA) is a novel 3-D hydrogel with various processing perspective that has been applied for biomedical applications and tissue engineering purposes. Cells attachment on the GelMA scaffold and encapsulated within the hydrogel matrix has been shown in previous studies (Nichol, et al., 2010). GelMA scaffold is widely applied to tissue engineering for clinical, diagnostic or pharmaceutical research purposes (Aubin, et al., 2010).

Aubin et al. (2010) grew different types of cells including HUVECs for vascularization, cardiac side population cells (CSPs) for myocardial tissue, and NIH 3T3 fibroblasts as a generic model on micro patterned GelMA to build different tissues (Aubin, et al., 2010).They demonstrated that by providing the micro-patterned guidance on hydrogel, vascular, muscle and cardiac cells could proliferate, align and elongate on GelMA. Accordingly, in order to manipulate the cellular micro-environment and control the cellular behaviors various tissue engineering studies have micro-patterned GelMA hydrogels (Nichol, et al., 2010; Aubin, et al., 2010; Nikkhah, et al., 2012).

The surrounding 3-D tissue scaffold needed to provide nutrients and oxygen for embedded cells and facilitate the resolving of metabolic byproducts (Chen, et al., 2012; Bertassoni, et al., 2014) Chen et al. (Chen, et al., 2012) and Lin et al. (Lin, et al., 2013) studied the formation of vascular network and they reported that GelMA hydrogel formed a stable substrate that caused an interconnected vascular distributed within the scaffold (Lin, et al., 2013). However, the tunable chemical and mechanical properties of GelMA hydrogel and biocompatible properties that reported previously encouraged me to develop GelMA 3-D scaffold for neuronal growth that has never been tested on neuronal tissue. This study is thus the first to test its utility for neuronal growth and axon-SC interactions.

5.5   neuronal Survivability and outgrowth of on GelMA

GelMA is produced by using gelatin at various concentration (5% and 10%) and a variety of crosslinking time under UV light. This intern functionalizes the functional group on gelatin and promotes the 3-D substrate for neuronal outgrowth (Van Den Bulcke, et al., 2000). Physical and mechanical properties such as porosity, stiffness, degradation, and cell response parameters like cell viability, proliferation, differentiation, survivability, neurite sprouting ability, neurite

extension length, neuron-SC interaction) on GelMA hydrogel are the key factors to illustrate that the hydrogel is a biocompatible scaffold for neuronal growth.

Neuronal sprouting on three types of substrate was studied by comparing phase contrast images. Figure 4C demonstrates that cell body of neuron seeded on GelMA is more flattened and spread out in all direction in a manner similar to that of PDL. These observations suggest that GelMA may enable grater contact surface contact resulting from firm attachment of the somta to the substrate and the inability of the somata to move away from the contact site during the pulling of the axons in various direction. Neurons adhered on the GelMA then extended their neurites and survived and continued to grow through at least 5 weeks in culture supporting the postulate that GelMA with microfibers and the porosity supported long-term survival (Mattson, et al., 2000). Phase contrast images of neuronal outgrowth on GelMA (Figure 3:15C) reveals that despite the surface porosity over GelMA, the neurites typically formed branches on hydrogel scaffold. This phenomenon can be attributed to the conducive properties of GelMA (Mattson, et al., 2000), while it has not been observed on PDL and collagen (Figure 3:15 A,B).

Comparison of neurons exhibiting outgrowth on collagen and GelMA was deemed essential to design a better substrate that facilitated both survival and growth. Even greater was the desire to find an appropriate substrate that could facilitate direct interactions between SC and axons in a manner analogous to that of *in vivo.*

Whereas the recent studies have focused on either soft or stiff mechanical properties of the substrate, the relationship between neurite outgrowth and substrate stiffness is not well stablished (Leach, et al., 2007). It is nevertheless evident that the stiffness of substrate impacts cells adhesion, proliferation, migration, differentiation and phenotype (Wong, et al., 2004; Zaari, et al., 2004). In this study, the relationship between substrate stiffness and neurite outgrowth was assessed. DRG

neurons seeded on 10% gelatin concentration GelMA with Yong modules 25 KPa (which is considered as the unit of stiffness of the hydrogel surface) enabled longer neurite outgrowth compared to collagen and PDL.

The texture of brain and spinal cord and peripheral nerves are reported (0.1-1 KPa) to be much softer than connective tissue with Yong's module (10-100 KPa) (Zaari, et al., 2004; Engler, et al., 2004). Therefor similarities of hydrogel stiffness to connective tissue is an important factor underlying the development of a suitable hydrogel scaffold for neuronal outgrowth. Based on my finding this is obvious that there exists a strong relation between the neurite behaviour and matrix stiffness, 1-neurite outgrowth and substrate stiffness have opposite ratio and 2- the maximal rate of neurite outgrowth occurs at an intermediate range of stiffness (Gunn, et al., 2005).

On the other hand, as SEM photographs demonstrated that the GelMA hydrogel with 10% gelatin concentration provided more contact surface available; neurons tend to attach firmly on the available substrate and extend their neurite through the smaller diameter pores. These randomly small diameter pore may have served as tunnels or channels through which individual axons traversed easily. Moreover, an alternative explanation would be that the axons preferred to adhere to this substrates and as such, did not opt to stick and fasciculate with each other.

5.6 Assessing the Myelination

After indicating the biocompatibility of GelMA hydrogel for DRG neuronal growth and reaching the single axons, the next step was to determine how the native SCs that accompanied the DRGs during the isolation procedures, interacted with individual axons. The SCs behavior on GelMA was obviously varied from those cultured on collagen and PDL which exhibited flattened shape

nuclei with approximately 150 μm process length. The length of SCs processes significantly increased on GelMA and remained viable for 5 weeks.

Between ECM adhesion receptors, integrins play key important role in producing intracellular signaling transduction required of growth-related signals (Juliano, et al., 2004), it has been reported that the expression of integrin is upregulated by contact of SCs with the DRG neurons (Einheber, et al., 1993).

During nerve regeneration and repair, the SCs behavior is effected by a variety of peptide sequences (Stabenfeldt, et al., 2006; Caprini, et al., 2013). The RGD sequence found in many ECM adhesive proteins plays a critical role in neurite outgrowth and SCs interaction. In this study, as GelMA contains the portion of RGD sequence, the interaction of RGD peptide with a variety of integrins can enhance the cell attachment.

When SCs come in contact with the single axons, they interact with them and myelination process can be investigated directly. As we know that when the precursor SCs (PSC) convert to mature or myelinating SCs that they are in close association with axons, their functions change and the nodes of Ranvier which are the clusters of Na^+ and K^+ channels are formed. To visualize the nodes of Ranvier *in vitro*, I used immunohistochemistry to stain node's protein including Nav1.6 and CASPR. The nodes protein were not detectable because they needed to be kept in culture more than 5 weeks which increased the chance of microbial contamination and substrate degradation. I then sought for the presence of Myelin protein which is easier to detect in culture. MBP and MAG were identified which strongly supported that some indicators of myelin may emerge in this model. See (Figure 4:10 and Figure 4:12).

5.7   Future directions

5.7.1   Tissue engineering application of GelMa

Whereas GelMA exhibited biocompatibility and produced single axons which were in contact with SCs and also the healthy behavior of SCs with extension of longer processes were also observed. A question thus arises whether GelMA hydrogel could be used as a potential scaffold for various tissue engineering applications.

In the context of promoting myelination in the PNS, SCs transplantation is being exploited for axonal regeneration after spinal cord injury. In the present study, biocompatibility properties of GelMA and also its structural and chemical versatility made it a suitable scaffold for neuron and SCs outgrowth. The high density of SCs survival on GelMA hydrogel and the quantification analysis for the length of processes by fluorescent microscopy, demonstrated that SCs when seeded on GelMA indicated the higher proliferation rate (Sedaghati, et al., 2014) compared to collagen and PDL.

The results of a study by (Nomura, et al., 2006) on different biodegradable and non-biodegradable polymers demonstrate that tissue engineering approaches exploring SCs may promote regeneration of spinal cord after injury. However, for a successful SCs transplantation and nerve repair a number of parameters ought to be considered (Nomura, et al., 2006; Yue, et al., 2015). It will be important to ensure that any nerve-electronics conduit may invoke emending of electrodes using GelMA. Its biocompatibility and the ability to keep axons separate may enhance nerve growth after injury. This substrate will also facilitate guided growth of injured axons – thus preventing mismatching and cross innervation which are the hallmarks of neuropathic pain.

5.7.2 Overall conclusion

The present study provided insights into developing a 3-D scaffold for neuronal outgrowth. DRG neurons were first seeded on PDL as 2-D substrate, they grew very well but did not allow interactions between DRG neuronal process and the native SCs. The PDL dishes thus served as control substrate against collagen. Collagen became a 3-D substrate for neuronal outgrowth, but because of axonal fasciculation that emerged out of somata, the SCs interactions with neurite were still unclear. Therefore, in order to have a 3-D scaffold that enabled neurons to extend their processes, a gelatin base hydrogel named GelMA, with tunable chemical and physical properties was applied. By manipulating various factors such as gelatin concentration, photo crosslinking time and UV intensity, the optimum condition for a biocompatible 3-D scaffold was obtained. I also examined the biocompatibility properties of GelMA by assessing the somata survivability, the ability of neurite extension, SCs survival and changes to their morphology and also the interaction between individual axonal neurites and SCs.

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